PHARMACOGNOSTICAL, PHYTOCHEMICAL AND *IN-VITRO* PHARMACOLOGICAL STUDIES ON THE STEM OF *MUSSAENDA FRONDOSA* LINN. RUBIACEAE.

By

SREENATH.R

Reg. No: 202780002

Thesis submitted to the Kerala University of Health Sciences in partial fulfillment of the requirements for the award of the Degree of

MASTER OF PHARMACY

IN

PHARMACOGNOSY

Under the guidance of

Smt. AJITHA H.S

Professor of Pharmacy

College of Pharmaceutical Sciences

Govt. Medical College, Thiruvananthapuram

FACULTY OF PHARMACEUTICAL SCIENCES

KERALA UNIVERSITY OF HEALTH SCIENCES

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JUNE- 2023

COLLEGE OF PHARMACEUTICAL SCIENCES
GOVT. MEDICAL COLLEGE
THIRUVANANTHAPURAM
PIN: 695011

DECLARATION

I undersigned here declare that the dissertation entitled ***"Pharmacognostical, Phytochemical and In-Vitro Pharmacological studies on the stem of Mussaenda frondosa Linn. Rubiaceae"*** *is a bonafide and genuine research work carried out by me, at College of Pharmaceutical Sciences, Govt. Medical College, Thiruvananthapuram, in the partial fulfilment of the requirements for the award of the Degree of* ***Master of Pharmacy in Pharmacognosy****, under the guidance of* ***Smt. AJITHA H.S****, Professor of Pharmacy, College of Pharmaceutical Sciences, Govt. Medical College, Thiruvananthapuram.*

Thiruvananthapuram
June 2023

SREENATH. R
College of Pharmaceutical Sciences,
Govt. Medical College,
Thiruvananthapuram

COLLEGE OF PHARMACEUTICAL SCIENCES
GOVT. MEDICAL COLLEGE
THIRUVANANTHAPURAM
PIN: 695011

CERTIFICATE

This is to certify that the investigation described in this thesis entitled ***"Pharmacognostical, Phytochemical and In-Vitro Pharmacological studies on the stem of Mussaenda frondosa Linn. Rubiaceae"*** *submitted by* ***Mr. SREENATH.R*** *in the partial fulfilment of the requirements for the award of degree of* ***Master of Pharmacy in Pharmacognosy*** *in the Faculty of Pharmaceutical Sciences, Kerala University of Health Sciences comprises the work done by him under my guidance and personal supervision in the Laboratories of College of Pharmaceutical Sciences, Govt. Medical College, Thiruvananthapuram.*

Smt. AJITHA. H.S
Professor of Pharmacy
College of Pharmaceutical Sciences
Govt. Medical College,
Thiruvananthapuram.

Thiruvananthapuram
June 2023

COLLEGE OF PHARMACEUTICAL SCIENCES
GOVT. MEDICAL COLLEGE
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Thiruvananthapuram
June 2023

Dr. Shini Dominic
Head of the Department
College of Pharmaceutical Sciences
Govt. Medical College,
Thiruvananthapuram

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Sreenath.R

College of Pharmaceutical Sciences,

Govt. Medical College,

Thiruvananthapuram.

June 2023
Thiruvananthapuram

"DEDICATED TO ALL THE

PHARMACY PROFESSIONALS

AND TO

THE WHOLE WORLD"

ABSTRACT

Mussaenda frondosa Linn. is a straggling bush belongs to the family Rubiaceae . It is also known as the 'White lady', found through out the world and mostly seen in all part of Kerala. Tribal peoples and various traditional systems of medicines uses different parts of this plant for the treatment of various diseases such as skin infections, fever, inflammation, jaundice etc. The present study was to carry out the Pharmacognostical, Phytochemical and In-vitro Pharmacological studies on the stem of *Mussaesnda frondosa* Linn, Rubiaceae. Pharmacognostical studies include Macroscopical and Microscopical studies of fresh and dried powdered stem. Physicochemical parameters like Ash value, Extractive value, Moisture content etc of powdered stem were determined. Two different type of extraction methods such as Maceration and Soxhlation were used in this study that helped to find out which method gives maximum yield. All the extracts were subjected to Preliminary Phytochemical screening. Quantitative estimation of Tannins, Flavonoids and Phenolic compounds were also done. In vitro antioxidant activity of all the extracts were determined by DPPH assay and the results were compared. Ethanolic extract showed maximum antioxidant activity compared to other extracts so it was selected for further pharmacological and chromatographic studies. Anti-oxidant, Anti-inflammatory and Anti-microbial activities of ethanolic extracts were determined according to standard procedures. Column chromatography of ethanolic extract was performed . Spectral studies of the isolated fraction were also done. Pharmacognostical and Physicochemical parameters helped to develop suitable standards of *Mussaenda frondosa* Linn. Phytochemical screening helped in the identification of various primary and secondary metabolites. In vitro Pharmacological studies helped in the evaluation of Pharmacological activities and thus conformation of therapeutic activity. Spectral studies given idea about the structure of the compound.

Key words: Pharmacognostical, Phytochemical, Chromatography,

In vitro Pharmacological.

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LIST OF SYMBOLS AND ABBREVIATIONS

cm	Centimeter
m	Meter
mm	millimeter
nm	Nanometer
g	Gram
kg	Kilogram
mg	Milligram
µg/mcg	Microgram
L	Litre
ml	Millilitre
µl	Microlitre
N	Normality
M	Molarity
mM	Millimolar
v/v	Volume/volume
w/v	Weight/volume

w/w	Weight/weight
min	Minute
hr	Hour
sec	Seconds
lbs	pound per square inch
^{0}C	Degree celsius
%	Percentage
GAE	Gallic acid equivalents
TPC	Total phenolic content
IC	Inhibitory concentration
MIC	Minimum Inhibitory Concentration
e.g.	Example
Abs	Absorbance
OD	Optical density
pH	Potential of Hydrogen
SEM	Standard error of mean
Rf	Retention factor
PBS	Phosphate buffer saline
BHA	Butylated hydroxyanisole
BSA	Bovine serum albumin
HepG2	Hepatoblastoma cell line
DMSO	Dimethyl sulfoxide

H2SO4	Sulphuric acid
HCl	Hydrochloric acid
HNO3	Nitric acid
KOH	Potassium hydroxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
NaOH	Sodium hydroxide
UV	Ultra-violet
FT-IR	Fourier transform infra-red
HPTLC	High performance Thin layer chromatography
HPLC	High performance liquid chromatography
TLC	Thin layer chromatography
GCMS	Gas chromatography Mass Spectroscopy

INTRODUCTION

INTRODUCTION

Medical history from the beginning of time is filled with descriptions of persons who used herbs to heal the sick of the society. The use of herbs as medicine is the oldest form of healthcare known to humanity and has been used in all cultures throughout history (Barnes et al., 2007). Early humans recognized their dependence on nature for a healthy life and since that time humanity has depended on the diversity of plant resources for food, clothing, shelter, and medicine to cure myriads of ailments.[1]

Plants, in particular, have been focused in the traditional medicine, dating from around 2600 BCE, recording 1000 plant-based product in Mesopotamia, which incorporate the utilization of extracted oils of *Cupressus arizonica* Greene, *Commiphora acuminata* Mattick, *Cedrus libani* A. Rich., *Glycyrrhiza glabra* L., and *Papaver somniferum* L. are still utilized for the treatment of common colds and coughs to swelling and parasitic diseases . Egyptian pharmaceutical which is around 2900 BCE old, claims the ancient practice with regard to conventional medicine. "Ebers Papyrus" is the best-recognized record dating from 1500 BCE, enrolling more than 700 drugs, often derived from plant sources. The Chinese traditional medicine has been extensively known throughout the centuries, with the record from around 2000 years back (1100 BCE; Wu Shi Er Bing Fang; containing 52 medicines), tailed by the herbal Shennong (100 BCE; 365 medicines) and the herbal Tang (659 CE; 850 medicines). However, the records of the Indian traditional medicine dates from before 5000 years (Charaka and Sushruta Samhitas hold with 341 and 395 herbal medicines separately 1000 BCE ago)]. On the other hand, the Romans and Greeks also added extensively to the coherent development of the use of traditional medicine in the ancient Western world (Dioscorides, a Greek physician ~100 CE), documented the collection, storage, and proper use of traditional medicine in the then "known world." However, parallel to the onset of the industrial revolution we witnessed the rise of allopathic medicine. Herbal medicine was also an effective healing method, but was viewed less enthusiastically. [2, 3]

Herbal medicines still cover up about 70–80% primary healthcare treatment of persons in the developing country of the world. The use of herbal

medicine becoming popular due to toxicity and side effects of allopathic medicines. This led to sudden increase in the number of herbal drug manufactures.

The percentage for primary health-care treatment with herbal medicine is high because of the general belief that herbal drug has no side effect instead of being cheap and easily available[4]

Present scenario

Currently, herbal remedies are used to treat both acute and chronic illnesses, as well as a wide range of diseases and conditions, including inflammation, depression, prostate issues, cardiovascular disease, and prostate difficulties, to name a few. In Africa, the Africa flower (traditional herbal medicine) has long been used in Africa to alleviate the wasting symptoms brought on by HIV.

Now, it is believed that nature contributes about 90% to the new drug molecule. Nature has provided many effective drug agent such as dactinomycin, bleomycin, and doxorubicin, vinblastine, irinotecan, topotecan, etoposide, and paclitaxel (anticancer), mefloquine chloroquine, amodiaquine artemisinin, dihydroartemisinin, artemether, and arteether (antimalarial), metformin and eventually the other biguanide, harunganin, cryptolepine, and maprouneacin (antidiabetic), and calanolide A, cucrcumin, phenethyl isocyanate, and phenoxidiol (anti-HIV drugs).[5]

Herbs can be viewed as biosynthetic chemical laboratories, producing a number of chemical compounds. Herbal remedies or medicines consist of portions of plants or unpurified plant extracts containing several constituents, which often work together synergistically.

It is one of the most essential and demanding tasks for scientists working in herbal drug development to investigate the efficacy of herbal medicine, to scrutinize adverse effects, to identify marker compounds or therapeutic agents in medicinal or botanical and to serious contaminants from herbal mixtures. Most important reasons or causes of botanical or herbal drug toxicity are improper identification or authentication of Botanical or herbals, improper or mislabeling of plant material, contamination of herbals with microorganisms, contamination of herbals with fungal toxins such as aflatoxin, contamination of herbals with pesticides and heavy metals, interaction with conventional drugs upon concomitant intake, improper or unprofessional processing and inadequate standardization.. Survey says only 10% of herbals in global market are standardized with special reference to

marker or active principles and their quality control parameters. Majority of the herbals or plant derived natural products which were use by the majority of population's needs strict development of standardization and quality control parameters. Most of these of plants are far away from its safety data or having very little information regarding their toxicity. [6]

FUTURE PROSPECTS IN HERBAL MEDICINES

At the moment, scientific research on medicinal plants is being carried out most intensely in research institutes, universities and pharmaceutical laboratories as well as in the clinics of many developed countries. This scientific study of medicinal plants is currently being conducted at a high level. The focus of this research is mostly in two directions. First, research is done on the active components of plants that have long been recognised for having therapeutic benefits. . The second area of fundamental research focuses on the exploration of previously unexplored remote places of the planet in quest of novel therapeutic plants and pharmaceuticals. Searching for new biologically active compounds from natural sources starts, obviously, in the field. Plant, microbial or animal materials to be sought and investigated may be selected through a number of approaches

Advances in biotechnology, particularly methods for culturing plant cells and tissues, should provide new means for the commercial processing of even rare plants and the chemicals they produce. These new technologies will extend and enhance the usefulness of plants as renewable resources of valuable chemicals. In the future, biologically active plant-derived chemicals can be expected to play an increasingly significant role in the commercial development of new products for regulating plant growth and for insect and weed control.[7]

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Ethno botanical information

Anvar K and Jazir Haneef ; (2015) in their study *"Ethnobotanical Plants Used For Postnatal Care By Traditional Practitioners From Kozhikode District, Kerala, India"*, reported that *Mussaenda frondosa* Linn. Leaf Juice/Extract used for post natal care, as a lactagoge,[8]

Sheeba C. V., Meena K. Cheruvathur; (2020) in their study *"Ethno Medicinal Practices of Tribes of Muthanga, Wayanad District of Kerala, India"* reported that Paniya Tribes of Muthanga, Wayanad District of Kerala, India, use *Mussaenda frondosa* leaves and stem as Hair wash[9]

Praveenkumar. K ; (2018) in his study *"Floristic Diversity of Puliyanamkunnu, Chalavara Grama Panchayath, Palakkad District, Kerala State"* has reported that, *Mussaenda frondosa* Linn. is a Red listed Tree which is used in Ayurveda, Siddha, Unani, Folk medicines [10]

Smita Bhat, G. S. Mulgund ; (2019) in their study *"Ethnomedicinal Plants Used For The Treatment Of Diabetes Mellitus In Siddapur Taluk Of Uttara Kannada District, Karnataka, India"* has reported that Mussaenda frondosa (Root) along with Justicia adathoda (Root) and Salacia reticulate(Root) were used in diabetes mellitus as Paste/ Oral Decoction/ Oral[11]

Asma, V. M. and Afidam, K. ; (2013), in their study "*Documentation Of Medicinal Plants Of Selected Sacred Groves Of Trichur District"* reported that *Mussaenda frondosa* Linn. whole plant is used in Cough, Bronchitis, fever, Inflammations, Ulcers, Leucoderma, Pruritus, Ophthalmopathy, Jaundice, Uropathy.[12]

Neelam Kumar ; (2014) in his study "*Some Medicinal Plants Of Tehsil Joginder Nagar, District Mandi, H.P., India*," reported that *Mussaenda frondosa* Linn. Whole plant, flowers, stem, shoot and root, is used in Demulcent, white leprosy, eye troubles, leucoderma, white leaves are given with milk in jaundice.[13]

2.2 Pharmacognostical studies

Shanthi S ; (2021) in her study "*Pharmacognostical studies on leaves of Mussaenda frondosa* Linn" reported Pharmacognostical, Physico-chemical, phytochemical and HPTLC studies of *Mussaenda frondosa* provide data to construct standardization protocol.[14]

Dr. D. Sukumar, B. Anandhi ; (2014) in their study "*Phytochemical And Antimicrobial Investigations On Mussaenda glabrata*", has reported that the fresh flowers have been found to contain the flavonol glycoside rutin and the structure of the isolated yellow pigment has been characterized by means of modern physical methods like UV, 1H-NMR, 13C-NMR, chemical reactions, hydrolytic studies and chromatographic investigations. The isolated rutin is found to contain considerable anti-inflammatory activity.[15]

Manasa D. J, Chandrashekar K. R ; (2022) in their study *"Detection Of Phenolic Compounds In Mussaenda Frondosa Linn. By UHPLC Coupled With QTOF"* reported that in the leaves, the UHPLC analysis showed the presence of seventeen phenolic acids and four terpenes at varying concentrations in different extraction solvents. Major compounds detected were resveratrol, sinapic alcohol, chlorogenic acid, rutin, protocatechuic acid, spiraeoside, protocatechuic aldehyde, asiatic acid, medicagenic acid, diosmin and esculetin; remaining were the minor compounds. Aqueous extract was found to be the best extraction solvent for all the detected compounds, however the chloroform was found to be the best for cinnamic acid derivatives. This is the pioneering report on the detection of all these bioactive principles except rutin and quercitin in *Mussaenda frondosa* Linn. [16]

2.3 Pharmacological studies

Sankhadip Bose, Sudip Kumar Manda, Purba Das *et al* ; (2020) in their study "*Comparative Evaluation of Anti-inflammatory, Antipyretic and Analgesic Properties of Ixora coccinea and Mussaenda frondosa (Rubiaceae) Leaves*" reported that the anti-inflammatory, analgesic, and antipyretic properties of methanol extracts of Ixora coccinea and *Mussaenda frondosa* Linn leaves were examined in a variety of animal (rodent) models, including carrageenan-induced paw edema, hot-plate, tail flick, acetic acid-induced writhing method, and brewer's yeast-induced pyrexia. The two plant's' methanol leaf preparations have strong analgesic and anti-inflammatory

properties. The study's findings suggest that *Ixora coccinea* and *Mussaenda frondosa's* leaf preparations in methanol have antipyretic properties[17]

Muruganandam , Rajesh. V *et al* ; (2016) in their study *"Antioxidant and Antidiabetic potentials of Mussaenda tomentosa Wight Ex Hook. F"* reported that the preliminary qualitative phytochemical screening of *Mussaenda tomentosa* fresh leaves has revealed for the presence of Phenols, Flavanoids, Glycosides,Terpenoids, Tannins, Reducing sugars, Proteins and absence of Alkaloids in methanol extract. The quantitative determination of phenol and flavanoids was carried out and found that the total phenolic content as 79.30 mg GAE/gram and flavonoids 36.49 mg QE/gram respectively. The IC50 value of DPPH assay in methanol extract was recorded as 80.33µg/mL. The Phosphomolybdenum assay and reducing power assay indicated that the plant extract has a potential antioxidant compared with the standard drug ascorbic acid. The IC50 value for the antidiabetic assay in methanol extract were recorded as 364.03 µg/ml and non-enzymatic glycosylation of hemoglobin shows 62% - 94% in the concentration of 250 - 1500 µg/mL. The inhibitory activity of Glucose uptake by yeast cells in methanol extract of *Mussaenda tomentosa* was found and compared with the standard drug acarbose.[18]

S. Shanthi, R. Radha ; (2020) in their study *"Anti-microbial and Phytochemical Studies of Mussaenda frondosa Linn. Leaves"* reported that the antimicrobial activity of Methanol, Ethyl acetate, Chloroform and Hexane extracts of *Mussaenda frondosa* Linn leaves were tested against nine bacteria such as Gram positive organisms- (Coagulase negative Staphylococcus, Staphylococcus aureus) and Gram negative organisms (Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhi, Salmonella paratyphi A, Salmonella paratyphi B and Vibrio cholerae) and four fungal strains such as Trichophyton simii, Trichophyton mentagrophytes, Aspergillus niger, Rhizopus and Candida albicans . The Methanol extract showed significant antibacterial and antifungal activity than hexane, Chloroform, Ethyl acetate extracts, which could be attributed to the presence of phenols, flavonoids and the other bioactive compounds identified through phytochemical screening[19]

Leena K. Pappachen, K. S. Sreelakshmi ; (2017) in their study *"Phytochemical Screening and In vitro Cytotoxicity Studies of Mussaenda frondosa*

Linn Leaves" reported that The methanolic extract of the leaves of *Mussaenda frondosa* Linn showed the presence of chemical constituents like flavanoids, phenolic compounds etc which was isolated by using HPTLC using solvent system Chloroform: ethylacetate (7.5:2.5) and Ethyl acetate: 1,4-Dioxane: Formic acid: Water (5:3:1:1) respectively. Both the methanolic extract and isolated flavonoid fraction was found to possess in vitro cytotoxic activity on HepG2 celline.[20]

Kavitha V.P, Anju Joseph *et al;* (2010) in their study "In Vitro Antioxidant Activity Of *Mussaenda frondosa"* reported that In vitro antioxidant effects of the ethyl alcohol and aqueous extracts of whole plant of *Mussaenda frondosa* Linn were tested. The ethyl alcohol extract of *Mussaenda frondosa* had shown good DPPH(1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity. The ethyl alcohol and aqueous extract of *Mussaenda frondosa* also exhibited promising result at higher concentration. BHA was used as standard antioxidant and positive control. The DPPH radical scavenging activity of the extract was increased with the increasing concentration .The reducing power of extract was carried out with ascorbic acid as a standard reducing agent. All the analysis was made with the use of UV-Visible spectrophotometer(Shimadzu 1700,INDIA).The study suggest that the *Mussaenda frondosa* could be pharmaceutically exploited for antioxidant properties.[21]

S. Gopalakrishnan and Vadivel. E ; (2011) In their study *"GC-MS Analysis Of Some Bioactive Constituents Of Mussaenda Frondosa Linn"* reported that the ethanolic extract of *Mussaenda frondosa* has been subjected to GC-MS analysis. Twenty chemical constituents have been identified, The major chemical constituents are (-)-Quinic acid (32.87 %), 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol (8.30%), Naphthalene, decahydro2-methoxy-(7.20 %). 1, 2, 3-Benzenetriol (7.70%).[22]

Chandrashekar Konambi Ramaiah, Manasa Dogganal Jayappa *et al* ; (2020) in their study *"Green synthesis of zinc oxide nanoparticles from the leaf, stem and in vitro grown callus of Mussaenda frondosa L.: characterization and their applications"* reported the first time synthesis of ZnO-NPs from callus, leaf and stem extracts of Mussaenda frondosa and asynthesized nanoparticles were characterized (XRD, UV–Vis, SEM, EDS, FTIR and DLS). In addition, the work also demonstrated a significant antioxidant, anti-inflammatory, antidiabetic and anticancer activities of ZnO-NPs.[23]

Suhas A. P and Joshi V. G ; (2011) in their study *"Evaluation of antibacterial and wound healing activity of leaves of Mussaenda frondosa linn"* reported that presence of flavonoids, steroids saponin, resin in various extracts was confirmed by preliminary phytochemical investigation, TLC, HPTLC, IR and Mass spectroscopic methods. The wound healing activity of *Mussaenda frondosa* Linn was evaluated by using different types of wound healing models such as excision wound, incision wound and dead space wound. All results were significant for different parameters in wound healing activity in alcoholic extract treated animals compared with control groups.[24]

Patil P. A, Kangralkar V. A. *et al* ; (2010) in their study *"Protective activity of Mussaenda frondosa leaf extracts against paracetamol induced hepatic damage in wistar rats."* reported that phytochemical analysis of aqueous & alcoholic extracts showed the presence of flavanoids, sterols, proteins & glycosides,aqueous extract showed the presence of flavanoids, proteins & glycosides while chloroform and petroleum ether extract showed only presence of carbohydrates and glycosides and no flavanoids and sterols. Study clearly indicate that both aqueous and alcoholic extracts of Mussaenda frondosa showed significant Hepatoprotective activity against paracetamol induced hepatic injury.[25]

Basavaraja, B. M., Vagdevi, H. M. *et al* ; (2011) in their study *"Antimicrobial and analgesic activities of various extracts of Mussaenda frondosa L. bark."* reported that The bark of the *Mussaenda frondosa* Linn was extracted with different solvents and screened for their antimicrobial and analgesic activities. Antimicrobial activity was evaluated using MIC method and analgesic activity was carried out by acetic acid induced writhing method. Methanol extract exhibited almost equipotent anti-bacterial activity as compared with that of standard drug ciproflaxacin and exhibited potent anti-fungal activity compared with that of standard clotrimazole. Chloroform extract of *Mussaenda frondosa* Linn bark has almost same percentage protection as compared with the standard drug acetyl salicylic acid in analgesic activity.[26]

Wesley. J. J, Ansar. A. M *et al* ; (2009) in their study *"Hypolipidemic effect of methanolic extract of Mussaenda frondosa Linn. leaves in high fat diet fed rats."* reported thatthe hypolipidemic effect of Methanolic extract of *Mussaenda*

frondosa may be due to the inhibition of hepatic cholesterogenesis and may have slight antioxidant activity. It is therefore concluded that the methanolic extract of *Mussaenda frondosa* possesses hypolipidemic activity in high fat diet fed rats.[27]

2.4 PLANT INTRODUCTION

THE GENUS MUSSAENDA

The genus *Mussaenda* is native to West Africa and seen through out the Indian sub-continent, South East Asia and Southern China. Important species like *Mussaenda frondosa, Mussaenda incana, Mussaenda macrophylla, and Mussaenda roxburghiii* are widely distributed in Central Nepal, India, and Sri Lanka. *Mussaenda raiatensis,* a native species of Tonga, occurs in open places on ridges, in coastal to lowland forests, and *Mussaenda pubescens* was discovered in shady hillside, valley, and shrub jungle of East, South, and Southwest China.. *Mussaenda hossei, Mussaenda Multinervis, Mussaenda erosa, Mussaenda laxiflora* were native to china [28]

The plant known as *Mussaenda frondossa* Linn family Rubiacea is a straggling bush that has been claimed to have a variety of therapeutic characteristics, including jaundice, asthma, hyperacidity, fever, ulcer, leprosy and a diuretic are just a few of the conditions historically treated with the plant. The leaf extract was found to have antimicrobial, diuretic, hepatoprotective, antipyretic, asthma, and cough properties. Mucilage, steroids, glycosides, saponins, resin, and flavonoids are all found in the leaves of *Mussaenda frondossa* Linn.[29]

Fig No. 1: *Mussaenda Frondosa* Linn

Fig No. 2: Berries

Fig No. 3: Calyx lobe formed as a white leaf like structure

Fig No. 4: Flowers and leaves

Synonym of *Mussaenda frondossa* Linn[30]

- *Gardenia frondosa* (L.) Lam
- *Mussaenda fruticosa* L.
- *Mussaenda dovinia Buch.*-Ham.
- *Mussaenda tomentosa* Wight Ex Hook. F
- *Mussaenda ingrata* Wall. ex Hook.f.
- Mussaenda flavescens Buch.-Ham.
- Mussaenda formosa L. nom. Illeg
- Mussaenda macrophylla Kurz nom. Illeg
- Mussaenda sumatrensis B.Heyne ex Roth

- *Mussaenda villosa* Schltdl. ex Hook.f. nom. illeg.

Table no. 1: Vernacular name of *Mussaenda frondosa* Linn:[29, 32]

Sanskrit	Srivati, Sriparnah, Sreeparna, Rajatari, Sreepatri, Sreemati, Shrivati
Tamil	Vellai-yilai, Vellimandai
Hindi	Bedina.
Malayalam	Vellila, Vellilathali
Telugu	Nagavalli
Kannada	Bellotti
Marathi	bhutakesha, sarvad
Konkani	sarvadi
Assamese	sona-rupa
Bengali	Nagaballi
English	Dhobi Tree, flag bush, White Lady, White flag bush, Schizomussaenda, white rag plant, wild mussaenda

Table no. 2: Scientific classification of *Mussaenda frondossa* Linn[31]

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida-Dicotyledons
Family	Rubiaceae
Genus	Mussaenda
Species	frondosa

Table no. 3: Botanical Description[30, 32, 33]

Habitat	Moist deciduous and semi evergreen forests, also in the plains
Flowering and fruiting	April- November
Leaves	Simple, opposite, broadly ovate, caudate acuminate at apex, base rounded, petiole 1 to 1.5 cm long; stipules 3-4 mm long. Cymes terminal linear
Flowers	5-merous. Bracts and bracteoles linear, Calyx-lobes 5, lanceolate, one of the calyx lobes transformed into a showy, white, leaf-like structure, Corolla 5-3 cm long, funnel-shaped dialated above middle, orange-red or yellow, villous at mouth; Stamens 5, included or exserted. Berry. 1 cm across, globose.

Chemical Constituents. [28, 29]

The phytoconstituents identified by GC MS analysis of ethanolic extract of Mussaenda frondosa has twenty chemical constituents. The major chemical constituents were identified as (-)-Quinic acid (32.87 %), 4-((1E)-3-Hydroxy-1propenyl)-2-methoxyphenol (8.30%), Naphthalene, decahydro-2-methoxy-(7.20 %). 1, 2, 3-Benzenetriol (7.70%). It has various pharmacological activities such as antiseptic, antioxidant, antidermatitic, fungicide, insecticide, antitumor etc.

Iridoids,flavanoids and triterpenes are the chemical ingredients distributed common Mussaenda species. Phytochemicals like isoquercetin, kaempferol-3-o-beta drutinoside in astragalin, were isolated from leaves. A new compound sanzhilactone along with mussaenside, barlerine, lupeol and beta-d glucose has been obtained from the stem. Quercetin, rutin, hyperin, ferulic acid, synaptic acid, beta sitosterol, saponins has also been isolated.

The flowers contain anthocyanins, hyperin, quercetin, ferulic, sinapic acid and beta-sitosterol glucoside

AIM AND PLAN OF WORK

3. AIM AND PLAN OF WORK

Mussaenda frondosa Linn. (Rubiaceae) commonly known as Wild Mussaenda or dhobi tree. It is found throughout India and has historically been used to treat a wide assortment of diseases. Aim of the present study was to carry out the pharmacognostical, phytochemical and in-vitro pharmacological studies on the stem of *Mussaenda frondosa* Linn

Pharmacognostical and physico-chemical parameters help to develop standards to determine purity and quality of the drug. Phytochemical screening helps to identify primary and secondary metabolites present in the extract. Chromatography is used for the isolation of bioactive constituents. Spectral analysis of these compounds gives information about its structure. In-vitro pharmacological studies helps in the confirmation of therapeutic activity.

Plan of work

1.Pharmacognostical study

a. Macroscopicl studies

b. Microscopical studies

2. Determination of Physico-chemical constants

a. Ash values

- Total ash
- Acid insoluble ash
- Water soluble ash
- Sulphated ash

b. Extractive values

- Water soluble extractive
- Alcohol soluble extractive
- Ether soluble extractive

c. Loss on drying

d. Determination of inorganic constituents

3. Fluorescence analysis of dried powder

4. Phytochemical Studies

a. Successive solvent extraction of powdered stem by Maceration and Soxhlet extraction using the following solvents-

- Petroleum Ether (60-80°c)
- Chloroform
- Ethyl acetate
- Ethanol
- Water

b. Preliminary phytochemical screening of all extracts.

c. Quantitative estimation of phyto-constituents.

d. Thin layer chromatography of all the extracts.

e. Adsorption column chromatography of the extract.

f. Spectral analysis of isolated compounds

- UV- Visible Spectroscopy
- FT-IR Spectroscopy
- HR-LCMS Spectroscopy

5. *In- vitro* Pharmacological screening

a. Anti-oxidant activity

b. Anti-inflammatory

c. Anti-microbial activity

- Anti-bacterial activity
- Anti-fungal activity

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1. PHARMACOGNOSTICAL STUDIES

4.1.1. Collection of specimens

The plant specimens for the proposed study were collected from Pachaloor, Thiruvanthapuram district of Kerala. The collected plant was examined and authenticated by the Dr. E.A. Siril, Professor and Head, Department of Botany, University of Kerala, Karyavattom Campus, Thiruvananthapuram. A voucher specimen (Voucher No: KUBH:11152) has been deposited for future reference.

4.1.2. Morphological characters [34, 35. 36, 37]

The morphological characters like colour, odour, taste, size, shape and other extra feature of the stem were studied, and the results were tabulated.

4.1.3. Microscopical studies[34, 35, 36,37]

The stems were subjected to various anatomical studies. The transverse sections of stem specimen were taken using sharp blade and stained with various staining reagents and observed under microscope Labomed, Binocular KXi 2000.

4.1.4. Preparation of powder [34, 35, 36, 37]

The collected stems of *Mussaenda frondosa* Linn were washed with running tap water to remove adhering materials. Then the stems were dried under shade. The dried stems were pulverized mechanically into coarse powder. The coarse powder (passed through sieve no. 18 and retained on sieve no. 60) used for Pharmacognostical and Phytochemical studies. The fine powder was used for Powder Microscopy.

4.1.5. Powder microscopy [34, 35, 36, 37]

Majority of the crude drugs for commercial purpose are available in powder form. So, the powder microscopic studies give the anatomical characters of fragmented crude drugs. Glycerin mounted temporary preparations were made for powdered stem and observed under microscope Labomed, Binocular KXi 2000.

4.2 DETERMINATION OF PHYSICOCHEMICAL CONSTANTS

4.2.1 Ash values [34, 35, 36, 37]

The ash of any organic material composed of their non-volatile inorganic components. Controlled incineration of crude drugs results in an ash residue consisting of an inorganic material (metallic salts and silica). Ashing involves oxidation of the components of the product. This value varies within wide limits and is therefore an important parameter for the purpose of evaluation of crude drugs. A high ash value is indicative of contamination, substitution, adulteration or carelessness in preparing the crude drug for marketing. In certain drugs, the percentage variation of the weight of ash from sample to sample is very small and any marked difference indicates a change in quality. Unwanted parts of drugs, sometimes, possess a character that will raise the ash value. More direct contamination, such as by sand or earth, is immediately detected by the ash value.

a. Total ash

Total ash is the measure of total amount of material produced after complete incineration of the ground drug at as low temperature as possible (about 450 °c) to remove all carbons. Total ash usually consists of carbonates, phosphates, silicates and silica which include both Physiological ash – derived from the plant tissue itself and Non-physiological ash – which is the residue of the adhering material to the plant surface, e.g., sand and soil.

Procedure

Incinerated about 3g accurately weighed powdered drug in tarred platinum crucible at a temperature not exceeding 450°c until free from carbon. Cooled and weighed, repeated to get constant value. Percentage total ash was calculated with reference to the air-dried drug.

b. Acid-insoluble ash

It is the ash obtained after extracting the total ash with Hydrochloric acid.

Procedure

The total ash obtained was boiled with 25 ml of 2 M HCl for 5 minutes. The insoluble matter was collected on an ashless filter paper (Whatman 41), washed with hot water. Transferred the filter paper containing insoluble matter to the original

crucible, ignited, weighed and calculated the percentage acid-insoluble ash with reference to the air-dried drug.

c. Water soluble ash

It is the part of total ash content which is soluble in water. It is a good indicator of either previous extraction of water-soluble salts in the drug or incorrect preparation. Thus, it is the difference in weight between the total ash and the residue obtained after treatment of total ash with water.

Procedure

The total ash obtained was boiled for 5 minutes with 25ml of water. Collected the insoluble matter on an ashless filter paper, washed with hot water, and ignited for 15 minutes at a temperature not exceeding 450°c. Subtracted the weight of the insoluble matter from the weight of the ash; the difference in weight represents the water soluble ash. Calculated the percentage of water-soluble ash with reference to the air-dried drug.

4.2.2 Extractive values [34, 35, 36, 37]

It is the amount of active constituents in a given amount of medicinal plant material when extracted with a particular solvent. The extraction of any crude drug with a solvent yields a solution containing different phyto-constituents. The composition of these phyto-constituents in that solvent depends upon the nature of the drug and solvent used. The use of a single solvent can be the means of providing preliminary information on the quality of a particular drug sample; for example, in a drug where the extraction procedure for the constituents commence with water as solvent, any subsequent aqueous extraction on the re-dried residue will give a very low yield of soluble matter. Water soluble, alcohol soluble and ether soluble extractive values are an indicative of poor quality, adulteration with any unwanted materials or incorrect processing of the crude drug during the process of drying, storage etc.

a.Alcohol soluble extractive

Macerated 5 g of air-dried, coarse powdered drug with 100 ml of 90% methanol in a closed flask for 24 hours, shaken frequently during the first 6 hours and allowed to stand for 18 hours. Then it was filtered rapidly, without any loss of solvent,

evaporated 25 ml of the filtrate to dryness in a tarred flat-bottomed shallow dish and dried at 105°c, to constant weight. Calculated the percentage of alcohol soluble extractive with reference to air dried drug.

b. Water soluble extractive

Macerated 5 g of the air dried, coarse powdered drug with 100 ml of Chloroform water in a closed flask for 24 hours, shaken frequently during the first 6 hours and allowed to stand for 18 hours. Then it was filtered, without any loss of solvent, evaporated 25 ml of the filtrate to dryness in a tarred flat bottomed shallow dish and dried at 105° c, to constant weight. Calculated the percentage water soluble extractive with reference to air dried drug.

c. Ether soluble extractive

Macerated 5 g of the air dried, coarse powdered drug with 100 ml of Diethyl ether in a closed flask for 24 hours, shaken frequently during the first 6 hours and allowed to stand for 18 hours. Then it was filtered, without any loss of solvent, evaporated 25 ml of the filtrate to dryness in a tarred flat bottomed shallow dish and dried at 105° c, to constant weight. Calculated the percentage ether soluble extractive with reference to air dried drug.

4.2.3 Loss on drying[32, 33, 34, 35]

It is the loss of weight expressed as percentage w/w. It determines the amount of volatile matter of any kind (including water) that can be driven off under the conditions specified.

Procedure

Accurately weighed about 1.5 g stem powder of *Mussaenda frondosa Linn.* in a tarred porcelain dish and dried in an oven at 105° c to constant weight, cooled in desiccator and weighed. From the difference in weights, percentage loss on drying was calculated.

4.2.4. Detection of inorganic constituents

To the prepared ash of the drug material, added 50% v/v Hydrochloric acid, kept for 1 hour and filtered. Then the following tests were performed on the filtrate.

a. Test for nitrates:

Warmed the filtrate with few drops of conc. sulphuric acid and copper turnings.

b. Test for chlorides:

To 3 ml of the filtrate added a few ml of 10% silver nitrate solution.

c. Test for carbonates:

2 ml of the filtrate was treated with few ml of mercuric chloride solution.

d. Test for sulphates:

A few ml of filtrate was treated with 5% barium chloride solution.

e. Test for Iron:

To 3 ml of the filtrate added a few ml of potassium ferrocyanide.

f. Test for Calcium:

To 3 ml of filtrate added few drops of aqueous ammonia to make the solution basic and added saturated ammonium oxalate solution drop wise.

g. Test for phosphates:

To the filtrate added nitric acid and a few drops of ammonium molybdate solution then heated for 10 minutes and cooled.

4.3 FLUORESCENCE ANALYSIS [38, 39]

Fluorescence is an important phenomenon exhibited by various chemical constituents. UV light produces Fluorescence in natural products which may not visibly fluorescent in day light. If the substances themselves are not fluorescent they may often be converted into Fluorescent derivatives by applying different reagents.

Procedure

The powdered stem of *Mussaenda frondosa* Linn was taken on a clean glass slide and treated with freshly prepared reagent. Mixed gently and observed under day light, UV light (short and long wavelength) to assess the fluorescent behavior of the drug and the results were recorded and tabulated.

4.4 PHYTOCHEMICAL STUDIES [40]

The whole plant or organism serves as an active laboratory for the production of natural products from primary metabolites. Primary metabolites are the products of vital metabolic pathways such as respiratory chain, TCA cycle etc. They are functional compounds found in virtually all plants. Secondary metabolites are

varieties of simple to sophisticated bizarre molecules. They are fascinating chemical molecules, very useful and of great importance in nature, as well as highly diversified in structures, properties, uses, chemistry etc.

Extraction

The process of separating active principles from powdered crude drugs by using suitable solvents is called Extraction. The choice of solvent depends upon the characteristics of the secondary metabolites like polarity, pH and thermal stability. Successive solvent extraction is suitable to extract the constituents of different polarity. It involves the extraction of the same plant material with solvents of different polarity ranging from non-polar to polar.

Successive Maceration [40, 41]

Maceration means to soften. It is an extraction process in which the drug powder is soaked in suitable solvent in a closed vessel for seven days with occasional shaking at room temperature. After specified period, the menstruum is strained and the marc is pressed to obtain the remaining menstruum. This marc is dried at a temperature not exceeding 50°c and is used for extraction with the next solvent.

Successive Soxhlet Extraction

Soxhlet extraction is also known as hot continuous percolation. Here the plant material is continuously flushed with fresh solvent which is obtained by evaporation and subsequent condensation of the solvent containing extracted materials. In successive soxhlation the plant material is extracted with solvents of increasing polarity and finally macerated with chloroform water. In the present study, dried powdered stem of *Mussaenda frondosa* Linn. was extracted with petroleum ether, chloroform, ethyl acetate, ethanol and water by successive maceration and soxhlet extraction. Before extracting with new solvent the marc is pressed and dried at a temperature not exceeding 50°c.

4.4.1 PREPARATION OF EXTRACTS [40,41]

Successive Maceration

Coarse powder of *Mussaenda frondosa Linn.* stem were extracted successively with the following solvents.

a. Petroleum ether extract

The coarse powder of stem of *Mussaenda frondosa* Linn. (50 g) was taken in a glass container and macerated with petroleum ether (60-80° c) for 7 days with occasional shaking and filtered. Then the extract was distilled under vacuum in order to remove the solvent completely and dried in desiccators. The extract was then weighed and calculated the percentage yield in terms of air dried crude material.

b. Chloroform extract

The marc left after straining the petroleum ether extract was dried in hot air oven below 50° c and then macerated with chloroform for 7 days with occasional shaking and filtered. Then the extract was distilled under vacuum in order to remove the solvent completely and dried in desiccators. The extract was then weighed and calculated the percentage yield in terms of air dried crude material.

c. Ethyl acetate extract

The marc left after straining the chloroform was dried completely in hot air oven below 50° c and then macerated with Ethyl acetate for 7 days with occasional shaking and filtered. Then the extract was distilled under vacuum in order to remove the solvent completely and dried in desiccators. The extract was then weighed and calculated the percentage yield in terms of air dried crude material.

d. Ethanol extract

The marc left after straining the ethyl acetate was dried completely in hot air oven below 50° c and then macerated with ethanol for 7 days with occasional shaking and filtered. Then the extract was distilled under vacuum in order to remove the solvent completely and dried in desiccator. The extract was then weighed and calculated the percentage yield in terms of air-dried material.

e. Aqueous extract

The marc left after straining the ethanol, extract was completely dried in hot air oven below 50° c and then macerated with distilled water for 7 days with occasional shaking and filtered. Then the extract was distilled under vacuum in order to remove the solvent completely and dried in desiccators. The extract was then weighed and calculated the percentage yield in terms of air-dried crude material.

Successive Soxhlet extraction

50 g of coarsely powdered drug was packed in Soxhlet apparatus and extracted using solvents such as petroleum ether (60-80°c), chloroform, ethyl acetate,

ethanol and finally macerated with chloroform water. The filtrate was concentrated in rotary vacuum evaporator and the extracts were weighed and calculated the percentage yield in terms of air dried material.

4.4.2 PRELIMINARY PHYTOCHEMICAL SCREENING [40]

Preliminary phytochemical screening was done to identify different classes of constituents present in extracts i.e. carbohydrates, proteins, lipids, flavonoids, tannins, glycosides, alkaloids, essential oils etc. All the extracts of *Mussaenda frondosa* Linn stem were subjected to preliminary phytochemical screening as per the standard procedures.

1. Detection of Carbohydrates:

- **Molisch's test:** 1 ml of the test solution was mixed with 2 ml of Molisch's reagent, shaken the mixture and added 1 ml of concentrated sulphuric acid along the sides of the test tube.
- **Benedict's test:** Mixed 2 ml of the Benedict's reagent with 2 ml of the test solution. Boiled in a water bath.
- **Fehling's test:** Boiled 1 ml of test solution with 1 ml Fehling's solution A and 1 ml Fehling's solution B on a water bath.
- **Barfoed's test:** Mixed 2 ml of the Barfoed's reagent with 1 ml of the test solution Boiled in a water bath.
- **Iodine test:** mixed 0.5 ml of Iodine solution with 1 ml of the test solution.

2. Detection of proteins and amino acids:

- **Biuret test:** About 2 ml of extract was mixed with 2 ml of Biuret reagent.
- **Millon's test:** About 2 ml of extract was mixed with 2 ml of Millon's reagent and boiled.
- **Xanthoprotein test:** 2 ml of the extract was treated with 1ml of concentrated Nitric acid and Sulphuric acid. Cooled the solution and made alkaline with 10% sodium hydroxide.

- **Ninhydrin test:** Boiled 2 ml of the extract with 1ml of 5% ninhydrin solution in a water bath for 5 minutes.

3. Detection of alkaloids:

- **Mayer's test:** 2 ml of the extract was treated with 2 ml of Mayer's reagent.
- **Dragendorff's test:** 2 ml of the extract was treated with 2 ml of Dragendorff's reagent
- **Hager's test:** 2 ml of the filtrate was treated with 1-2 ml of Hager's reagent
- **Wagner's reagent:** 2 ml of the filtrate was treated with 1-2 ml of Wagner's reagent.

4. Detection of glycosides

- **Borntrager's test:** To a little quantity of sample solution added Sulphuric acid and carbon tetrachloride. Separated the organic layer and shaken with dilute ammonia.
- **Modified Borntrager's test (modified anthraquinone test for C-glycosides):** To little quantity of sample solution added ferric chloride solution, hydrochloric acid and carbon tetrachloride. Separated the organic layer and shaken with dilute ammonia.
- **Baljet test:** mixed 2-3 mg of sample in 2 ml sodium picrate solution.

5. Detection of flavonoids

- **Shinoda test:** To 2 ml of the sample solution added magnesium powder and few drops of concentrated hydrochloric acid or sulphuric acid.
- **Sulphuric acid test:** Added few drops of concentrated sulphuric acid to few ml of sample solution.
- **Lead acetate test:** Mixed few ml of the test solution with lead acetate solution.
- **Alkali test:** Treated the test solution with increasing amount of sodium hydroxide.

6. Detection of tannins and phenolic compounds

- **Ferric chloride test:** Mixed 2 ml of the test solution with few ml of 5% ferric chloride solution.
- **Lead acetate test:** Mixed 2 ml of the test solution with 1 ml of lead acetate solution.
- **Dilute iodine test:** Mixed few ml of the test solution with dilute iodine solution.
- **Potassium dichromate test:** Mixed few ml of the test solution with potassium dichromate solution.
- **Dilute Nitric acid test:** Mixed few ml of the test solution with dilute nitric acid.
- **Bromine water test:** Mixed 2 ml of the test solution with bromine water.

7. Detection of steroids and triterpenoids

- **Libermann test:** Mixed 2 ml of test solution with 2 ml of acetic anhydride and boiled. Then added 0.5 ml of concentrated sulphuric acid.
- **Libermann-Buchard test:** Mixed 2 ml of the test extract with 1 ml of Chloroform and 1 ml acetic anhydride. Then added 1 drop of concentrated sulphuric acid.
- **Salkowski test:** Dissolved 1-2 mg of sample in 1 ml of chloroform and added 1 ml of concentrated sulphuric acid.

8. Detection of saponins

- **Foam test:** Shaken few gm of powder with 20 ml of distilled water.

9. Detection of mucilage

- **Swelling test:** Dissolved the powder in water

4.4.3 QUANTITATIVE ESTIMATIONS

a. Estimation of Total Flavonoid content [40, 41, 42, 43]

Principle

Flavanoids are plant pigments responsible for colour. It has antioxidant action, used in venous disorders, and for some infections. The principle of Aluminium

chloride colorimetric method is that aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavanols. In addition, aluminium chloride forms acid labile complexes with the ortho-dihydroxyl groups in the ring A or B of flavonoids.

Materials required

Standard Quercetin, Aluminium chloride solution (10 %), , Potassium acetate(1 M), Ethanol, Distilled water, Extracts.

Procedure

Preparation of standard solution

10 mg of quercetin was dissolved in methanol and make up to 10 ml. From the above solution (1 mg/ml), 1 ml was pipetted out and made up to 10 ml with methanol to get 100 mcg/ml of quercetin stock solution. From the stock solution, solutions of concentration 20, 40, 60, 80 and 100 µg/ml were prepared. 0.5ml of standard solution was added with 1.5 ml of 95 % ethanol. After that 0.1 ml of 10 % aqueous aluminium chloride solution was added followed by addition of 0.1 ml of 1 M Potassium acetate and 2.8 ml with distilled water. The solutions were mixed and absorbance was measured against the blank without addition of aluminium chloride at 415 nm using UV-Visible spectrometer. A standard graph was plotted using various concentrations and their corresponding absorbance.

Estimation of flavonoid content

Each extract (0.5 ml) was mixed with 1.5 ml of 95 % ethanol. and subsequently with 0.1 ml of 10 % aqueous aluminium chloride solution. After 5 minutes, 0.1 ml of 1 M Potassium acetate was added followed by addition of 2.8 ml with distilled water. The mixture was thoroughly mixed and absorbance was determined at 415 nm versus blank. Results were expressed as quercetin equivalents (mg quercetin/g of dried material). The estimation of flavonoid content was carried out in triplicate.

b. Estimation of Total Tannin content[40, 41, 42, 43, 44,45]

Principle

Tannin is an astringent, polyphenolic biomolecule that binds and precipitates proteins and various other organic compounds including amino acids and alkaloids. They are classified into hydrolysable and non- hydrolysable tannins.

Tannins reduce phosphotungsto-phosphomolybdate in alkaline solution to produce a highly blue colored solution, the intensity of which is proportional to amount of tannins. The intensity is measured in a spectrophotometer at 700 nm. The tannin content was expressed in terms of mg of tannic acid equivalents/g of dried sample.

Materials required

Folin- Ciocalteu reagent, Sodium carbonate solution (35 % w/v), Standard tannic acid, Distilled water, Extracts.

Procedure

The tannins were determined by Folin- Ciocalteu method. About 0.5 ml of the sample was added to volumetric flask containing 7.5 ml of distilled water and 0.5 ml Folin- Ciocalteu reagent, 1 ml of 35% Sodium carbonate solution and diluted to 10 ml with distilled water. The mixture was shaken well and kept at room temperature for 30 minutes. A set of reference standard solution of tannic acid (20, 40, 60, 80 and 100 µg/ml) were prepared in the same manner described earlier. Absorbance of test and standard solutions were measured against the blank at 700 nm with an UV/Visible spectrometer. The estimation of the tannin content was carried out in triplicate. The tannin content was expressed in terms of mg of tannic acid equivalents/g of dried sample.

c. Estimation of Total Phenolic content [40, 41, 42, 43, 44, 45]

Principle

Phenolics are secondary metabolites in plants derived from phenyl alanine and tyrosine. Mainly phenolics are simple phenols, phenolic acids (benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans, and lignin's which acts mainly as phytoalexins, attractants for pollinators, contributors to plant pigmentation, antioxidants, and protective agents against UV light, among others. The reaction of Folin – Ciocalteu reagent with polyphenols in the plant forms a blue chromophore constituted by a phosphotungsto-phosphomolybdenum complex where the maximum absorption of the chromophores depends on the alkaline solution and the concentration of phenolic compounds. It works by measuring the amount of the substance being tested needed to inhibit the oxidation of the reagent.

Materials required

Folin-Ciocalteu reagent, 7 % sodium carbonate solution, Extracts, Standard gallic acid, Distilled water.

Procedure

The total phenolic content (TPC) of extracts was estimated by Folin – Ciocalteu assay. It is also called Gallic Acid Equivalent method. 0.5 ml of sample was mixed with 0.5 ml of Folin –Ciocalteu phenol reagent. After 5 minutes, 5 ml of 7 % sodium carbonate solution was added to the mixture followed by the addition of 6.5 ml of distilled water and mixed thoroughly. The mixture was kept in the dark for 90 minutes at 28 °C, after which the absorbance was read at 760 nm. A set of reference standard solution of Gallic acid (20, 40, 60, 80 & 100 µg/ml) were prepared in the same manner described earlier. The total phenolic content was determined from extrapolation of calibration curve which was made by preparing Gallic acid solution. The estimation of phenolic compounds was carried out in triplicate. The TPC was expressed as milligrams of gallic acid equivalents (GAE)/g of dried sample.

4.4.4 THIN LAYER CHROMATOGRAPHY [46, 47, 48]

Plant extracts contains various types of bioactive compounds having different polarities. Their separation can be done using Thin Layer Chromatography (TLC). Samples were separated based on their interaction between a thin layers of adsorbent attached on the plate. In the present investigations, the studies were aimed at designing qualitative TLC parameters for various extracts of *Mussaenda frondosa* Linn and to find out suitable eluents for it.

Basic steps involved in TLC

a) Adsorbent selection and plate preparation

b) Solvent selection

c) Saturation of chromatographic chamber with solvent

d) Sample application as spots or bands over the chromatographic plate

e) Running the chromatogram

f) Detection

g) Qualitative and quantitative analysis

Procedure

Pre coated TLC silica gel G plates were activated by heating in hot air oven at 105° C for 1 hour. The extracts were dissolved in respective solvents and applied in TLC plates by means of capillary tube. The plates were then developed in TLC chamber previously saturated with different solvent systems. By trial and error method appropriate solvent system were developed and different spots developed were visualized by means of UV light and Rf values were calculated.

Rf value = Distance travelled by solute /Distance travelled by solvent front

4.4.5 COLUMN CHROMATOGRAPHY [49, 50]

The term column chromatography is used to refer to those methods in which the separation takes place within a packed column. The packing material was the stationary phase and was solid with adsorptive or exclusion capabilities. A liquid mobile phase is used as the eluent. Each compound in a mixture will have a particular solubility in the solvent and a tendency to be gets adsorbed by the solid adsorbent. No two compounds mostly behave exactly alike in these aspects. This principle is utilized in column chromatography. The technique used here was the isocratic elution technique.

Procedure

The column was filled with activated silica gel for column chromatography (60-120#) to a height of 37 cm in the solvent system itself. Column chromatography of ethanolic extract was done by using N- Hexane: Toluene: Ethyl acetate: Formic acid. (3: 5: 3: 0.6) using isocratic elution technique. 2 g of the extract was suspended in minimum quantity of solvent and triturated with small amount of silica on a mortar and pestle. The powder is then introduced at the top of the column and elution was carried out at the rate of 30 drops per minute. Several 10 ml fractions were collected and each fraction was checked by TLC. The fractions were grouped according to homogeneity judged from TLC analysis.

Table no: 4: Column chromatography of Ethanolic extract of stem of *Mussaenda frondosa Linn.*

Adsorbent	Silica gel for column chromatography 60-120#
Eluents	N-Hexane:Toluene:Ethyl acetate:Formic acid. (3:5:3:0.6)
Elution technique	Isocratic elution technique.
Length of column	50 cm
Diameter of column	2 cm
Amount of extract used	2 g
Length of column packed	37 cm
Rate of elution	30 drops per minute
Fraction collected	10 ml

4.4.6 SPECTRAL ANALYSIS OF ISOLATED COMPOUNDS[51, 52, 53]

a. UV-Vis spectroscopy

It is concerned with the study of absorption of UV radiation that ranges from 200 nm to 400 nm (2000 A° to 4000 A°). This region starts at the blue end of the visible light (about 4000 A°) and ends at 2000 A°. The UV region is subdivided into two spectral regions: Near UV region -200 nm to 400 nm and Far or vacuum UV region – below 200nm.

Compounds which are colourless absorb radiation in the UV region. In both UV and visible spectroscopy, only the valence electrons absorb energy. The UV radiation has sufficient energy to excite valance electrons in many atoms or molecules. When the valance electrons absorb the energy, the molecules undergo transition from ground state to excited state. This absorption characteristic depends on the concentration and path lengths as given by Beer- Lamberts law. The UV absorption spectroscopy study of the compound isolated from ethanolic extract of the *Mussaenda frondosa* Linn stem was carried out in College of Pharmaceutical Sciences, Thiruvananthapuram.

Instrument used -Hitachi model spectrophotometer

Model - UH5300

Solvent used - Ethyl acetate

Wave length - 200-800 nm

Response – Quick

b. FT-IR Spectroscopy

An FT-IR (Fourier transform Infra-Red) spectrometer obtains an infrared spectrum by first collecting an interferogram of a sample signal using an interferometer, then performs a Fourier Transform on the interferogram to obtain the spectrum. An Interferometer is an instrument that uses the technique of superimposing (interfering) two or more waves, to detect differences between them. The FT-IR spectrometer uses a Michelson interferometer. Fourier transform defines a relationship between a signal in time domain and its representation in frequency domain. Being a transform, no information is created or lost in the process, so the original signal can be recovered from the Fourier transform and vice versa. The Fourier transform of a signal is a continuous complex valued signal capable of representing really valued or complex valued continuous time signals. Fourier Transform Infra- Red spectrum of the fraction obtained from the column chromatography of the ethanolic extract of the stem and investigated for their characteristic functional groups.

Instrument used - Jasco FT- IR-4100

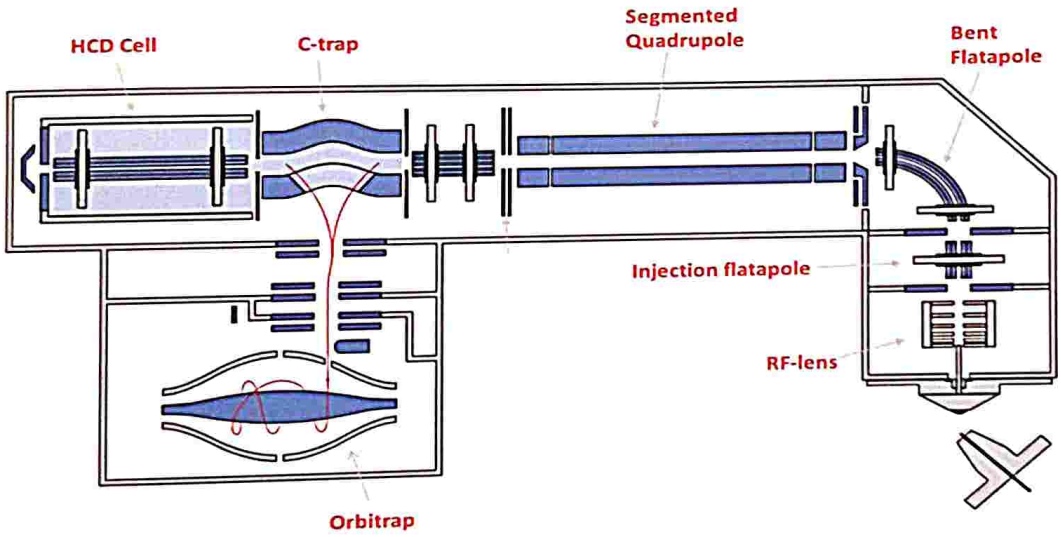
Wave number - 4000-650 cm-1

c) HR-LCMS ORBITRAP (High Resolution Orbitrap Liquid Chromatograph Mass Spectrometer)

Liquid chromatography / Mass Spectroscopy (LC / MS) is a technique which combines high performance liquid chromatography HPLC, a powerful analytical separation technique with mass spectroscopy,a powerful analysis & detection technique. There are two common atmospheric pressure ionization (API) LC/MS process: Electrospray Ionization (ESI) & Atmospheric Pressure Chemical Ionization (APCI). Both are soft ionization technique. Q Exactive Plus provides fast, sensitive, reliable and confident detection and identification of compounds in complex

mixture while maintaining full compatibility with UHPLC and fast chromatography. The HCD collision cell adds more functionality to the bench top system by providing Ion fragmentation while maintaining high resolution, accurate mass and high sensitivity.

The Orbitrap is an ion trap mass analyzer that consists of two outer electrodes and a central electrode, which enable it to act as both an analyzer and detector. Ions entering the Orbitrap are captured through "electrodynamic squeezing," after which they oscillate around the central electrode and in between the two outer electrodes. Different ions oscillate at different frequencies, resulting in their separation. By measuring the oscillation frequencies induced by ions on the outer electrodes, the mass spectra of the ions are acquired using image current detection.



Instrument used: Q-Exactive Plus Biopharma, Thermo Scientific

Data Acquisition Software: Thermo Scientific Xcalibur, Version 4.2.28.14

Data Processing Software: Compound Discoverer 3.2 SP1

Column details- SB-C18 RRHD 100 x 2.1 MM, 1.8 microns (Agilent Technologies).

Column temp: 40°c

Flow rate: 0.3ml/min

Run time: 35min

Solvent A: 0.1% formic acid in Milli-Q water, Solvent B: Methanol, Solvent C: Acetonitrile

Mass method: FULL MS / DD-MS^2 (TOPN) and dd-MS^2 / dd-SIM

Run time: 35 min

4.5 *IN-VITRO* PHARMACOLOGICAL STUDIES

4.5.1 *IN- VITRO* ANTI-OXIDANT ACTIVITY

DPPH (2, 2- diphenyl- 1- picryl hydrazyl) ASSAY [54, 55, 56, 57]

The radical scavenging activity of all the extracts of stem of *Mussaenda frondosa Linn.* were determined by using DPPH assay according to Chang et al (2001). The decrease in the absorption of DPPH solution after the addition of an antioxidant was measured at 517 nm. Ascorbic acid was used as reference.

Principle

2, 2-diphenyl-1-picrylhydrazyl is a stable free radical with red color which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity.

Antioxidants react with DPPH and reduce it to DPPH-H and as a consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

Reagent preparation

0.1 mM DPPH solution was prepared by dissolving 4 mg of DPPH in 100 ml of ethanol.

Procedure

0.1 mM solution of DPPH in ethanol was prepared and it was protected from light influence by maintaining the dark condition and also by covering the test tube with aluminium foil. 3 ml of this solution was added to 1 ml various concentrations (100-400 mcg/ml) of extracts and standard solution of ascorbic acid

(5-25 mcg/ml). Absorbance was taken after 30 minutes at 517 nm. The percentage inhibition activity was calculated.

$$Percentage\ inhibition = \frac{(OD\ of\ control - OD\ of\ test)}{OD\ of\ control} \times 100$$

Where, OD - Optical density

4.5.2 ANTI-INFLAMMATORY ACTIVITY [58, 59, 60]

Protein Denaturation Method

Materials required

Bovine serum albumin Fraction V- HiMedia, Hydrochloric acid-1N, Phosphate buffer, Distilled water

Procedure

Different concentrations of sample such as 62.5µg/mL- 500µg/mL from a stock solution of 10mg/mL were used for the study. The test control consists of bovine serum albumin and 0.05 mL of distilled water. The test solution consists of 0.45 mL of bovine serum albumin and different concentrations of sample. Diclofenac sodium was used as standard solution. All the above solutions were adjusted to pH 6.3 using 1N HCl. The samples were incubated at 37°C for 20 minutes and the temperature was increased to 57°C for 3 minutes. After cooling, 2.5ml of phosphate buffer was added to the solutions. The absorbance was measured using UV-Visible Spectrophotometer at 416nm (Cary win60- agilent).

CALCULATION

The percentage inhibition of protein denaturation can calculated as

$$Percentage\ inhibition = \frac{(OD\ of\ control - OD\ of\ test)}{OD\ of\ control} \times 100$$

Where, OD - Optical density

Statistical analysis

Statistical analysis was done by using one-way ANOVA followed by Turkey's test. P values lesser than 0.05 were considered as significant.

4.5.3 ANTIMICROBIAL ACTIVITY [61, 62, 63, 64]

Antimicrobial activity of plants can be detected by observing the growth response of various microorganisms to those plant tissues or extracts, which were placed in contact with them. In order to detect antimicrobial activity in plant extracts, three conditions must be fulfilled. First, the plant extract must be brought into contact with the cell wall of the microorganisms that have been selected for the test. Second, condition must be adjusted so that the microorganisms were able to grow when no antimicrobial agents were present. Third, there must be some means of judging the amount of growth, if any, made by test organism during the period of time chosen for the test.

A. Antibacterial Assay By Agar Well Diffusion Method

Principle

The antimicrobials present in the samples are allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The resulting zones of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeters.

Materials Required

1. Muller Hinton Agar Medium (1 L)

The medium was prepared by dissolving 33.8 g of the commercially available Muller Hinton Agar Medium (MHI Agar Media) in 1000ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto 100mm petriplates (25-30ml/plate) while still molten.

2. Nutrient broth (1L)

One litre of nutrient broth was prepared by dissolving 13 g of commercially available nutrient medium (HI Media) in 1000ml distilled water and boiled to dissolve the medium completely. The medium was dispensed as desired and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

3. Streptomycin (standard antibacterial agent, concentration: 10mg / ml)

4.Culture of test organisms; growth of culture adjusted according to McFarland Standard, 0.5% 1. *Staphylococcus aureus* (ATCC 25923).

Procedure

Petriplates containing 20ml Muller Hinton Agar Medium were seeded with bacterial culture of *Staphylococcus aureus* (growth of culture adjusted according to McFarland Standard, 0.5%). Wells of approximately 10mm was bored using a well cutter and different concentrations of sample such as 250µg, 500µg and 1000µg were added. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well (NCCLS, 1993). Streptomycin was used as a positive control.

B. Antifungal Assay By Agar Well Diffusion Method

Principle

In order to access the biological significance and ability of the sample, the antifungal activity was determined by Agar well diffusion method. The antifungals present in the samples are allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The resulting zones of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeters.

Materials Required

1) Potato Dextrose Agar Medium (1 L)

The medium was prepared by dissolving 39g of the commercially available Potato Dextrose Agar Medium (HiMedia) in 1000ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto 100mm petriplates (25-30ml/plate) while still molten.

2. Clotrimazole (standard antifungal agent, concentration: 10mg / ml)

3. Culture of test organisms; growth of culture adjusted according to McFarland Standard 0.5%, *Candida albicans* (ATCC 10231)

Procedure

Potato Dextrose agar plates were prepared and overnight grown species of fungus, *Candida albicans* were swabbed. Wells of approximately 10mm was bored using a well cutter and samples of different concentrations such as 250µg, 500µg and 1000µg were added. The zone of inhibition was measured after overnight incubation at room temperature and compared with that of standard antimycotic (Clotrimazole) (NCCLS, 1993).

Table no. 5 : Organisms, standard, medium and method used for anti-microbial studies.

	Anti-bacterial activity	**Anti-fungal activity**
Method	Agar well diffusion method	
Organism	*Staphylococcus aureus*	*Candida albicans*
ATCC No	25923	10231
Incubation condition	37°C for 24 hours	37°C for 24 hours
Standard	Streptomycin	Clotrimazole
Medium	Muller Hinton Agar medium	Potato Dextrose Agar

RESULT AND DISCUSSION

5. RESULT AND DISCUSSION

5.1 PHARMACOGNOSTICAL STUDIES

5.1.1 Morphological evaluation of fresh stem of *Mussaenda frondosa Linn.*

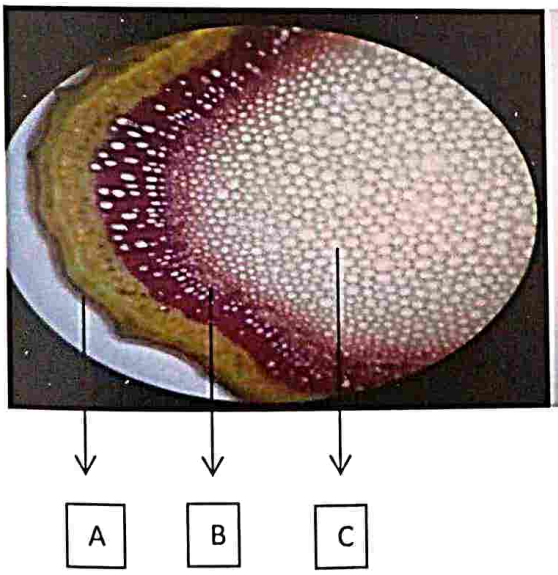
Fig No. 5: Stem of *Mussaenda frondosa* Linn.

Table no. 6: Morphological characters of *Mussaenda frondosa* Linn stem .

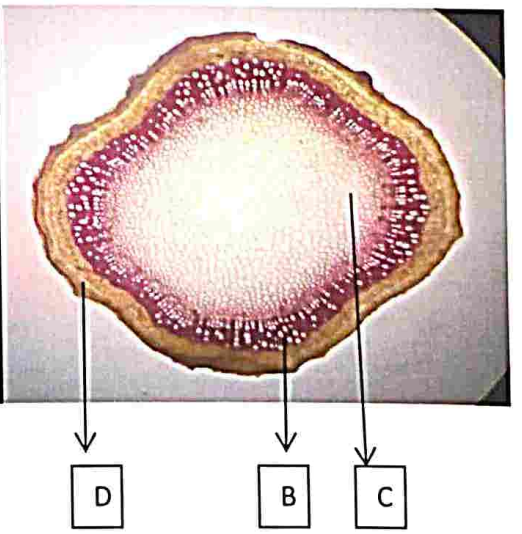
Sl no	Features	Observations
1.	Stem Colour	Dark green or brown,
2.	Stem Odour	Odourless
3.	Stem Taste	Tasteless
4.	Stem Shape	Round or square
5.	Size	Grow around 6-7 feet high
6.	Stem Texture	Hard woody, flexible

7.	Leaves	simple, opposite, broadly ovate, caudate acuminate at apex, petiole 1 to 1.5 cm long; stipules 3-4 mm long, ovate, bifid at apex.
8.	Flowers	Flowers 5-merous. Bracts and bracteoles linear, 1-1.5 cm, . Calyx-lobes 5, one of the calyx lobes transformed into a showy, white, leaf-like structure, Corolla 2.5-3 cm long, funnel-shaped dialated above middle, orange-red or yellow, tomentose outside, villous at mouth. Berry 1 cm across, globose

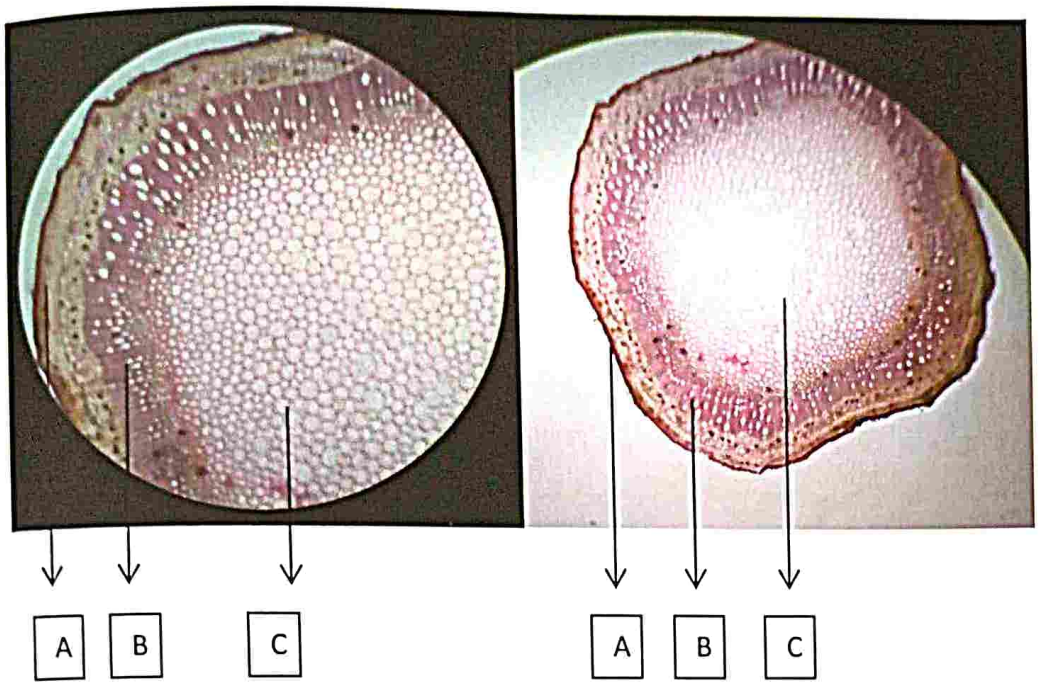
5.1.2 Microscopical examination of fresh young stem of *Mussaenda frondosa* Linn.



Staining reagent – Phloroglucinol
Magnification – 10x



Staining reagent – Phloroglucinol
Magnification – 4x

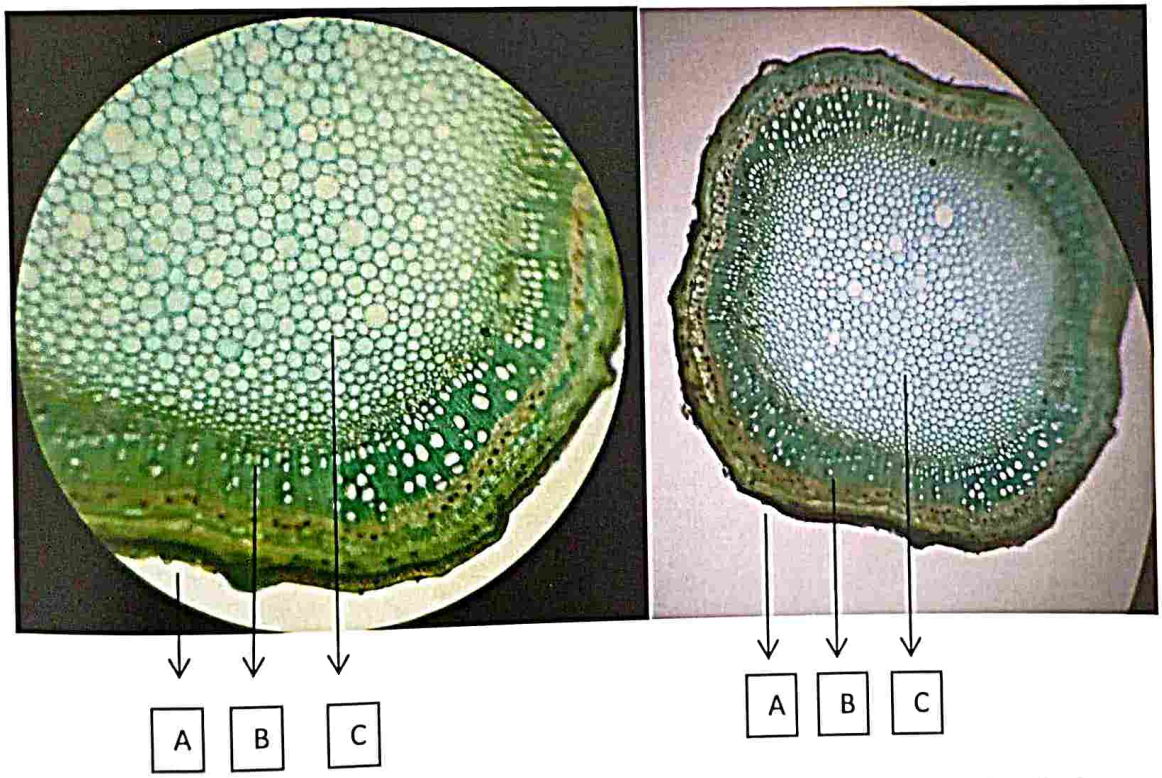


Staining reagent – Saffraine

Magnification – 10x

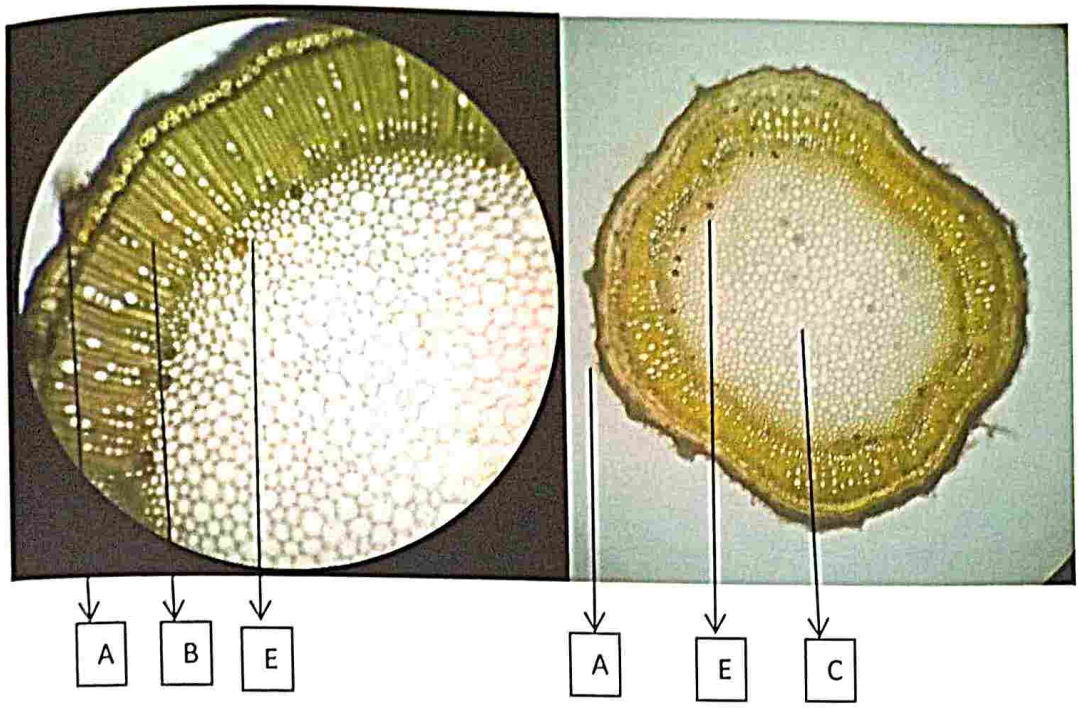
Staining reagent – Saffraine

Magnification – 4x



Staining reagent – Methylene Blue, Magnification – 10x

Staining reagent – Methylene Blue, Magnification – 4x



Staining reagent – iodine

Magnification – 10x

Staining reagent – iodine

Magnification – 4x

Fig No. 6: Transverse section of *Mussaenda frondosa* Linn stem section stained with Phloroglucinol, Saffraine, Methylene Blue, iodine. Under magnification 4x and 10x. A- Cuticle, B- Xylem and Phloem, C- Pith, D- Cortex, E- Oil Glands

5.1.3 Microscopical examination of dried powder of *Mussaenda frondosa Linn.*

Fibre

Parenchyma cell

Fibre

Xylem

Fig No. 7: Powder microscopical observations of *Mussaenda frondosa* Linn stained with Phloroglucinol Under magnification 40x

5.2 PHYSICOCHEMICAL CONSTANTS

5.2.1 Determination of physicochemical constants of *Mussaenda frondosa Linn.*

Table no. 7 : Physicochemical constants of *Mussaenda frondosa* Linn

Sl. No	Physicochemical constants	Average %w/w
1.	**Ash values:**	
	a) Total ash	3.55 ± 0.111
	b) Acid insoluble ash	0.5 ± 0.111
	c) Water soluble ash	1.3
2.	**Extractive values:**	
	a) Ether soluble extractive	2.4
	b) Alcohol soluble extractive	6.66 ± 0.4619
	c) Water soluble extractive	7.4 ± 1.6653
3.	Loss on drying	2.14 ± 0.319

Values were expressed as Mean ± SEM, n=3

5.2.2 Detection of inorganic constituents present in the ash of *Mussaenda frondosa Linn.*

Table no. 8 :Chemical tests for the detection of inorganic constituents

Sl.no	Experiment	Inference
1.	Test for Nitrates	Absent
2.	Test for Carbonates	Absent
3.	Test for Chlorides	Present
4.	Test for Sulphates	Present
5.	Test for Iron	Absent
6.	Test for Calcium	Absent
7.	Test for Phosphates	Absent

5.3 FLUORESCENCE ANALYSIS

Table no. 9 : Fluorescence analysis of powdered stem of *Mussaenda frondosa Linn.*

Sl. No:	Treatment	Day light	UV 254nm	UV 365nm
1.	Dry powder	Light/whitish brown	Light brown	Brown
2.	Powder + 1N HCl	Whitish brown	Greenish yellow	Fluroscent orange
3.	Powder + 1N H_2SO_4	Whitish brown	Greenish yellow	Fluroscent orange
4.	Powder + 1N HNO_3	Whitish brown	Greenish yellow	Fluroscent orange
5.	Powder + 1N NaOH	Reddish brown	Greenish yellow	Brown
6.	Powder + Alcoholic KOH	Reddish brown	Greenish yellow	Dark orange/brown
7.	Powder +Picric acid	Yellow	Greenish yellow	Black
8.	Powder + 5% $FeCl_3$	Light brown	Greenish brown	Black
9.	Powder+5% iodine	Black/dark brown	Black/dark brown	Black
10.	Powder+ DilKMnO4	Brown	Light brown	Dark Brown
11.	Powder +Acetic acid	Yellowish brown	Greenish yellow	Fluroscent orange
12.	Powder + Acetone	Yellow	Greenish yellow	Fluroscent orange
13.	Powder + Ethanol	Yellow	Greenish yellow	Fluroscent orange
14.	Powder+50%	Light brown	Greenish	Brown

	H2SO4		yellow	
15.	Powder + ConH2SO4	Dark brown/black	Dark brown/black	Black
16.	Powder + Con HCl	Brown	Brown	Brown dark
17.	Powder + ConHNO3	Reddish brown	Yellowish brown	Black
18.	Powder + Dil. NH3	Reddish brown	Greenish yellow	Dark brown
19.	Powder + Na2CO3	Reddish brown	Greenish yellow	Brown

5.4 PHYTOCHEMICAL STUDIES

5.4.1 Successive solvent extraction –Maceration and Soxhlet extraction

Fig No. 8: Soxhlet extraction

Table no. 10: Colour and Nature of Various Extracts of *Mussaenda frondosa* Linn. by Successive Maceration and Soxhlet Extraction.

SL.No.	Extracts	Colour	Nature	% yield(% W/W)	
				Maceration	Soxhlation
1.	Petroleum ether	Dark green	Sticky	0.9	3.45
2.	Chloroform	Dark green	Sticky	0.52	1.74
3.	Ethyl acetate	Dark green	Gummy	0.36	0.9
4.	Ethanol	Dark reddish brown	Sticky	1.76	2.52
5.	Water	Dark reddish brown	Gummy	1.42	4.95

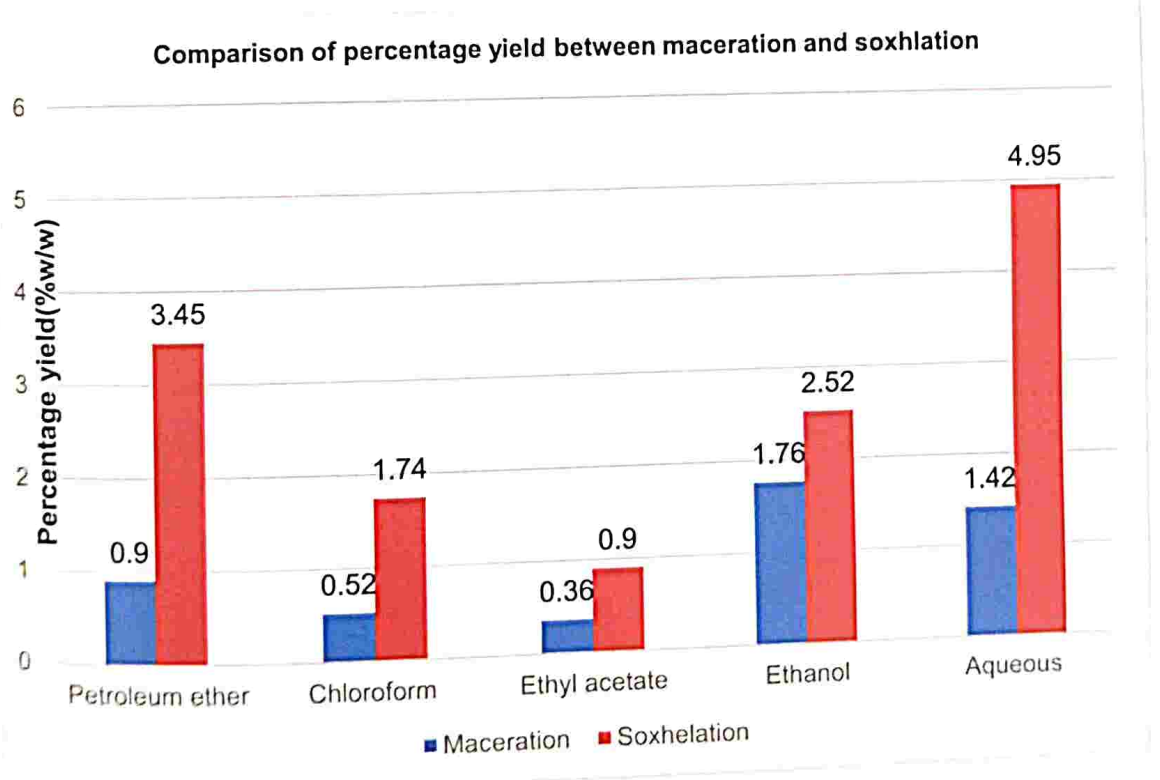


Fig No. 9: Comparative percentage yield of various extracts by Maceration and Soxhlation.

5.4.2 Qualitative phytochemical analysis

Table no. 11 : Phytochemical screening of various extracts of *Mussaenda frondosa* Linn.

Chemical constituents	Tests	Petroleum ether extract	Chloroform extract	Ethyl acetate extract	Ethanol extract	Aqueous extract
Alkaloids	Mayer's test	-	-	-	-	-
	Dragendorff's test	-	-	-	-	-
	Hager's test	-	-	-	-	-
	Wagner's test	-	-	-	-	-
Carbohydrate	Molisch's test	-	+	+	+	-
	Fehling's test	-	+	+	+	-
	Benedict's test	-	+	+	+	-
	Barfoed's test	-	+	+	+	-
	Iodine test	-	-	-	-	-
Protein and amino acids	Biuret test	-	-	-	-	-
	Millon's test	-	-	-	-	-
	Ninhydrin test	-	-	-	-	-
	Xanthoprotein test	-	-	-	-	-
Steroids and Triterpenoids	Liebermann test	+	+	+	-	-
	Liebermann-Burchard test	+	+	+	-	-
	Salkowski test	+	+	+	-	-
Fixed oils and fats	Filter paper test	-	-	-	-	-
Glycosides	Borntrager's test	-	-	-	-	-
	Modified Borntrager's test	-	-	-	-	-
	Baljet test	-	+	+	-	-
Tannins – phenolic compounds	Ferric chloride test	-	-	-	+	+
	Lead acetate test	-	-	-	+	+

	Potassium dichromate test	-	-	-	--	-
	Dilute HNO_3 test	-	-	-	-	-
	Dilute iodine test	-	-	-	-	-
	Bromine water test	-	-	-	-	-
		-	-	-	-	-
Flavonoids	Shinoda test	-	+	+	-	-
	Sulphuric acid test	-	+	+	-	-
	Lead acetate test	-	+	+	-	-
	Alkali test	-	+	+	+	-
Saponins (on Powder)	Foam test	-				
Mucilege (on Powder)	Swelling test	-				

Table no. 12 : Observation of phytochemical screening

Extract	Maceration	Soxhlet extraction
Petroleum ether	Steroid	Steroid
Chloroform	Steroid, Carbohydrates, Flavanoids, Glycoside	Steroid, Carbohydrates, Flavanoids, Glycoside
Ethyl acetate	Steroid, Carbohydrates, Flavanoids, Glycoside	Steroid, Carbohydrates, Flavanoids, Glycoside
Ethanol	Carbohydrates, Flavanoid, Tannins	Carbohydrates, Flavanoid, Tannins
Aqueous	Tannins	Tannins

5.4.3. Quantitative estimation of phytoconstituents

a. Estimation of total Flavonoids in *Mussaenda frondosa Linn.* – Aluminium chloride colorimetric method.

Table no. 13: Absorbance of standard quercetin and extracts of *Mussaenda frondosa* Linn.

Sl No.	Concentration (µg/ml)	Mean absorbance (415 nm)
1.	20	0.0905±0.0185
2.	40	0.157±0.025
3.	60	0.226±0.03
4.	80	0.315±0.037
5.	100	0.4055±0.0485
6.	*Mussaenda frondosa* Linn of Ethyl acetate extract (500µg/ml)	0.1775±0.0125
7.	Chloroform extract *Mussaenda frondosa* Linn.(500µg/ml)	0.2945±0.0265
8.	Ethanolic extract of *Mussaenda frondosa* Linn.(500µg/ml)	0.120±0.0021

Values are expressed in Mean ± SEM, n = 3

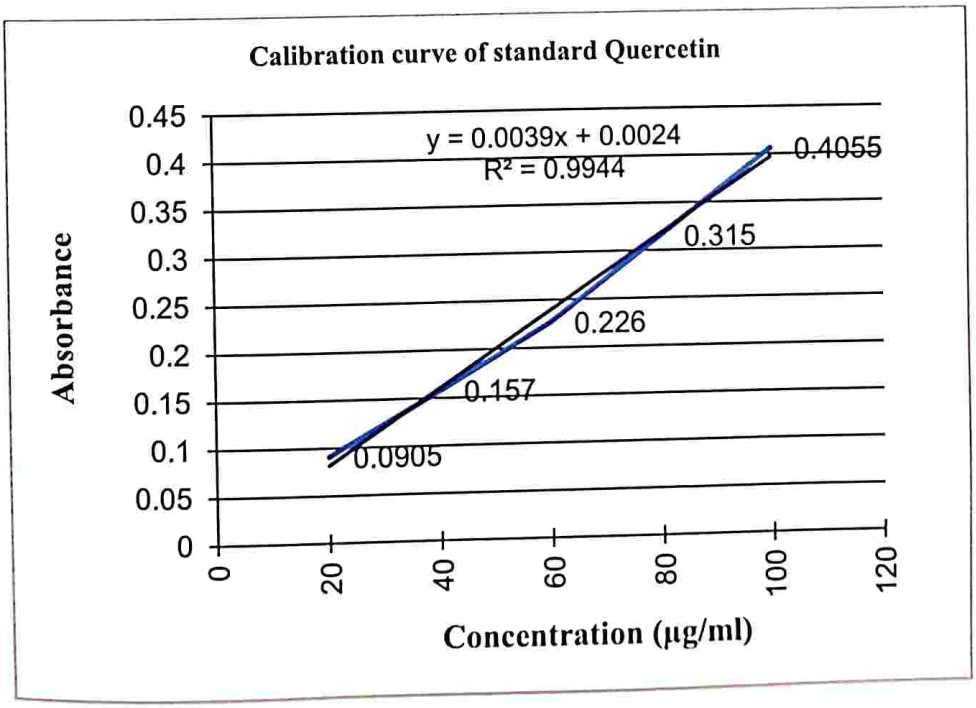


Fig No. 10: Calibration curve of Quercetin

Table no. 14 : Total Flavanoid content of chloroform, ethyl acetate and ethanol extracts of *Mussaenda frondosa Linn.*

Sl. No.	Extract (500 µg/ml)	Total Flavanoid content Quercetin equivalent to µg/ml
1.	Ethyl acetate extract	44.44
2.	Chloroform extract	74.13
3.	Ethanolic extract	29.51

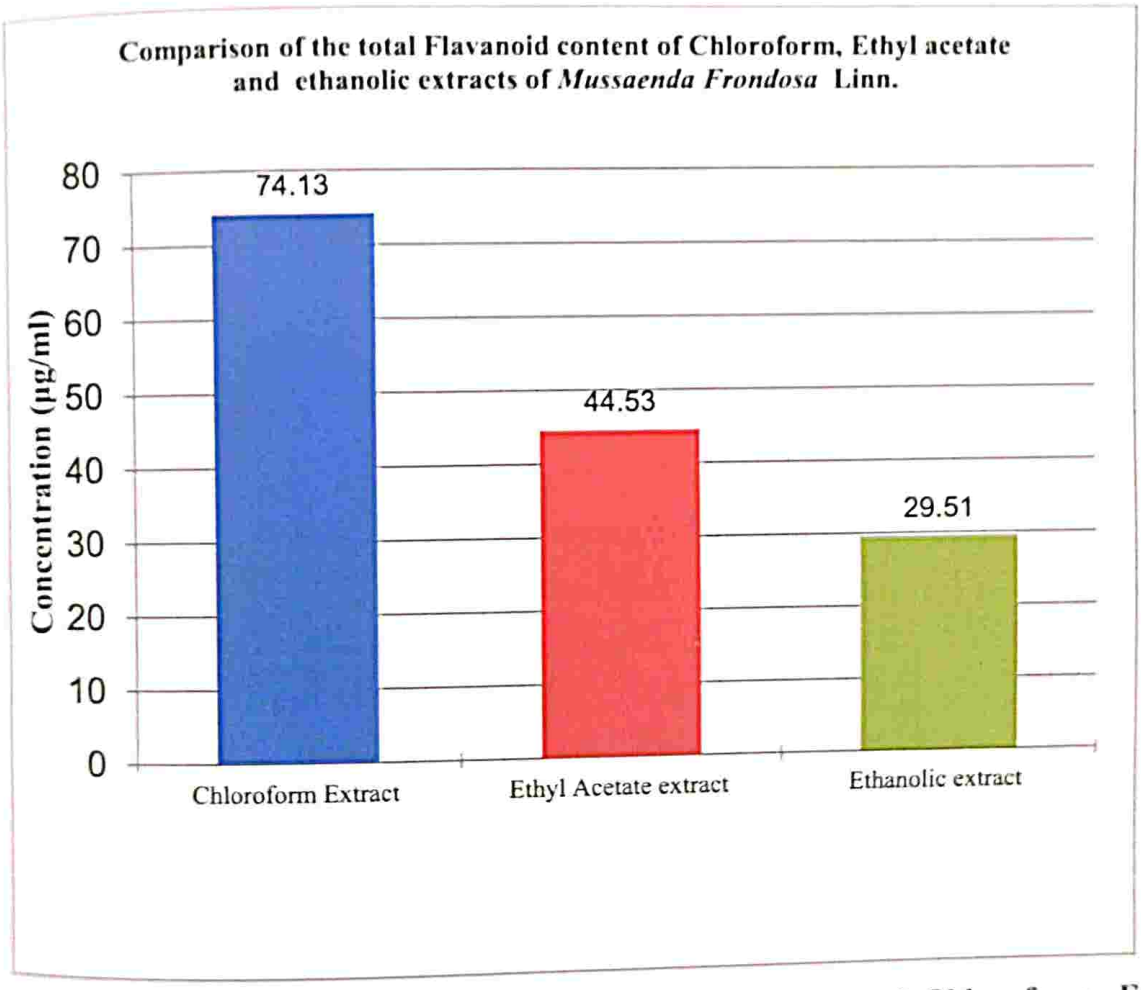


Fig No. 11: Comparison of the total Flavanoid content of Chloroform, Ethyl acetate and Ethanolic extracts of *Mussaenda frondosa* Linn.

b. Estimation of total Tannin content in *Mussaenda frondosa* Linn. by Folin's Denis method

Table no. 15: Absorbance of standard tannic acid and extract of *Mussaenda frondosa* Linn..

Sl. No	Concentration (µg/ml)	Mean absorbance (700 nm)
1.	20	0.0935±0.0025
2.	40	0.183± 0.012
3	60	0.258± 0.028
4.	80	0.3265± 0.0135
5.	100	0.421± 0.036
6.	*Mussaenda frondosa* Linn aqueous extract (400µg/ml)	0.1065± 0.0175
7.	*Mussaenda frondosa* Linn ethanolic extract (400µg/ml)	0.169± 0.024

Values were expressed as Mean ± SEM, n=3

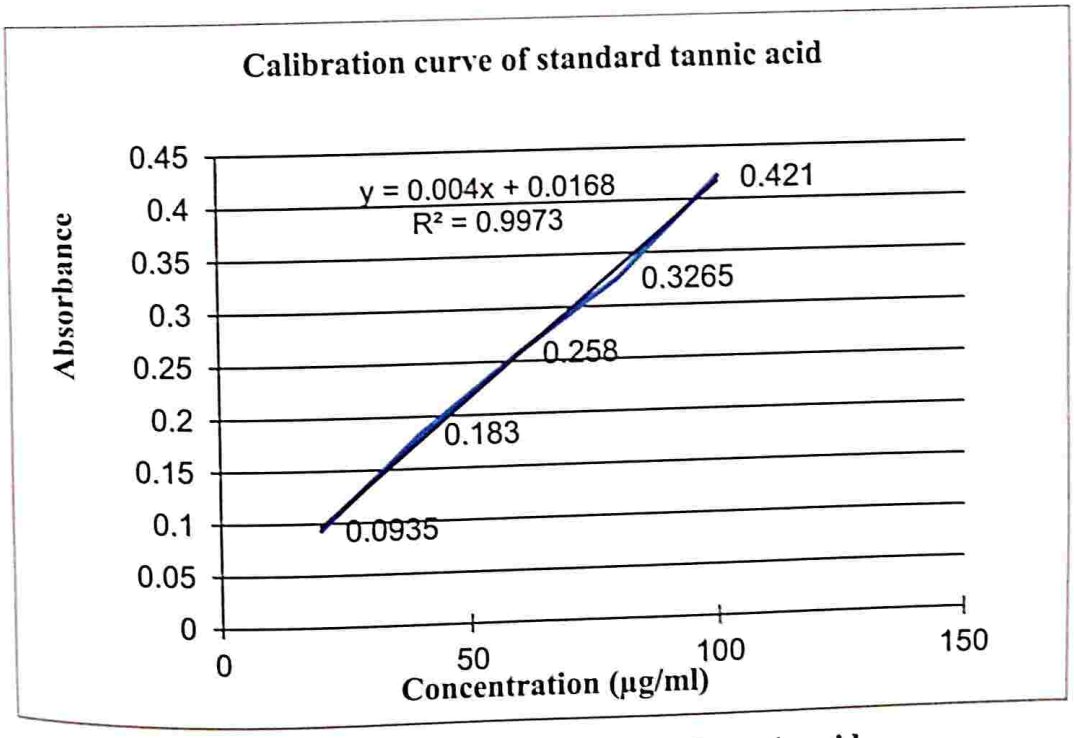


Fig No.12:Calibration curve of standard tannic acid

Table no. 16: Total tannin content of Aqueous and ethanolic extract of *Mussaenda frondosa* Linn.

Sl no:	Extract (400 µg/ml)	Tannin content- Tannic acid equivalent to µg/ml
1.	Aqueous extract	22.56
2.	Ethanolic extract	38.17

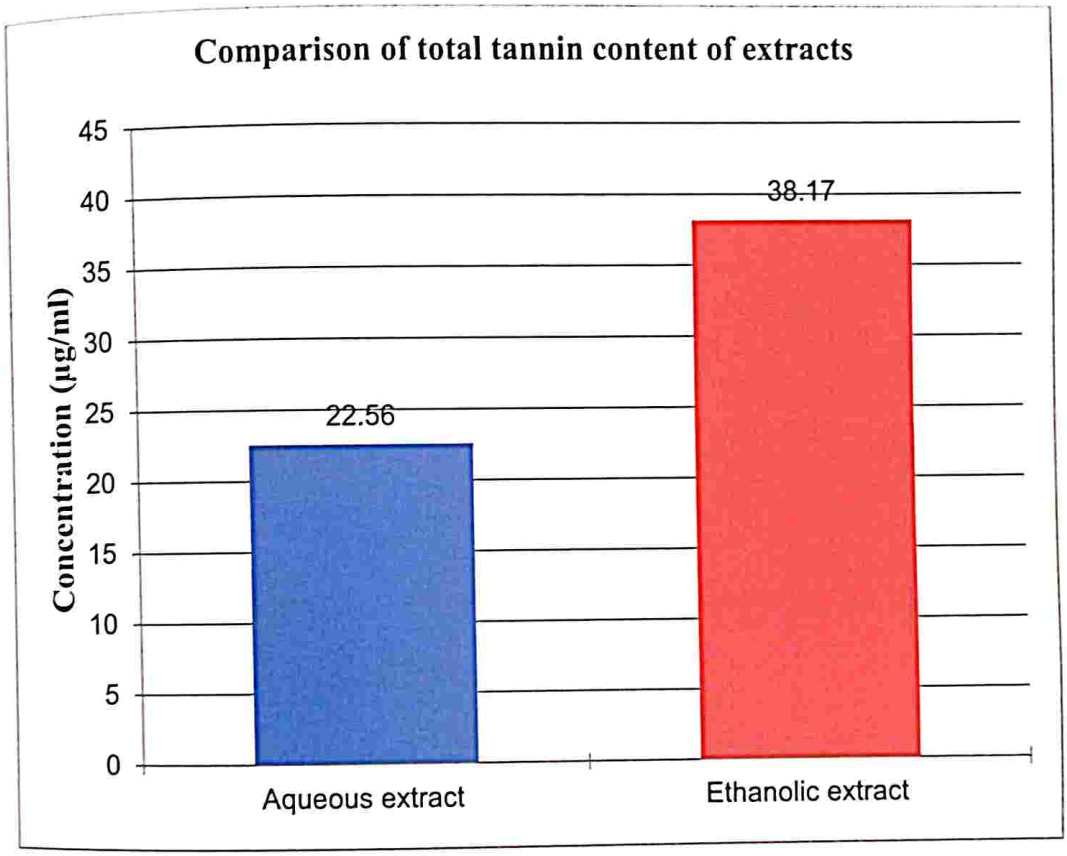


Fig No. 13: Comparison of the Total Tannin content of Aqueous and ethanolic extract of *Mussaenda frondosa* Linn.

c. Estimation of total Phenolic content in *Mussaenda frondosa* Linn. – Folin's Ciocalteu method / Gallic Acid Equivalent method

Table no. 17: Absorbance of standard gallic acid and extract of *Mussaenda frondosa* Linn.

Sl No.	Concentration (µg/ml)	Mean absorbance (760 nm)
1.	20	0.102 ± 0.0025
2.	40	0.208± 0.012
3.	60	0.3205± 0.028
4.	80	0.4035± 0.0135
5.	100	0.5135± 0.036
6.	*Mussaenda frondosa* Linn aqueous extract (500µg/ml)	0.212± 0.042
7.	*Mussaenda frondosa* Linn ethanolic extract (500µg/ml)	0.306±0.024

Values were expressed as Mean ± SEM, n=3

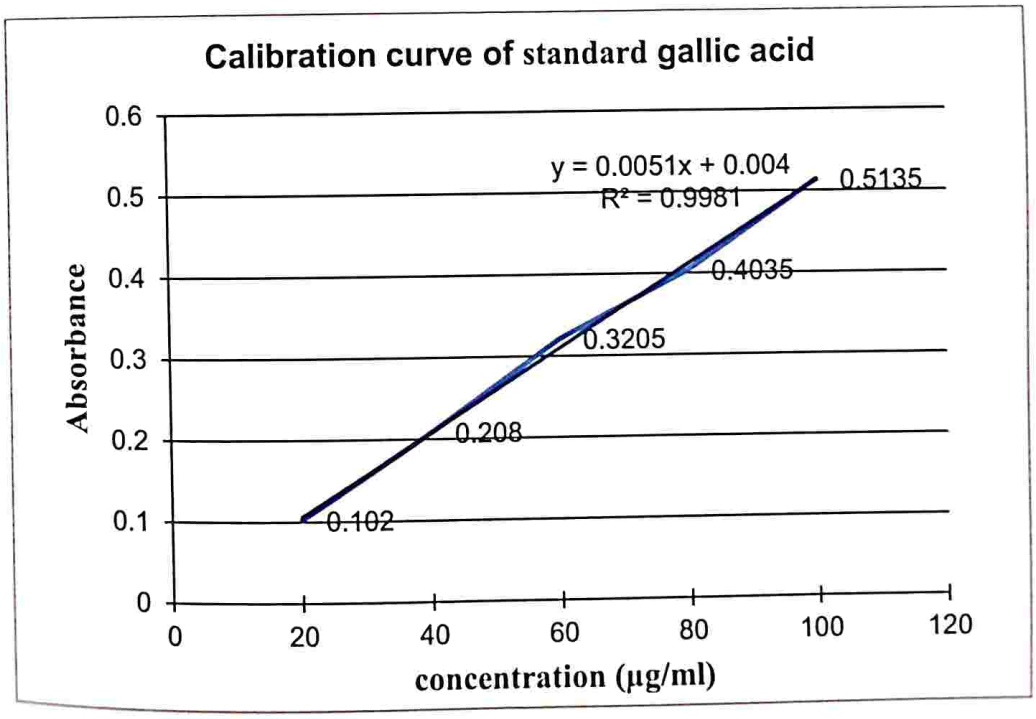


Fig No 14.: Calibration curve of standard gallic acid

Table no. 18 : Total phenolic content of Aqueous and ethanolic extract of *Mussaenda frondosa* Linn.

Sl. No	Extract (500µg/ml)	Phenolic content- gallic acid equivalent to µg/ml
1.	Aqueous extract	40.9
2.	Ethanolic extract	59.31

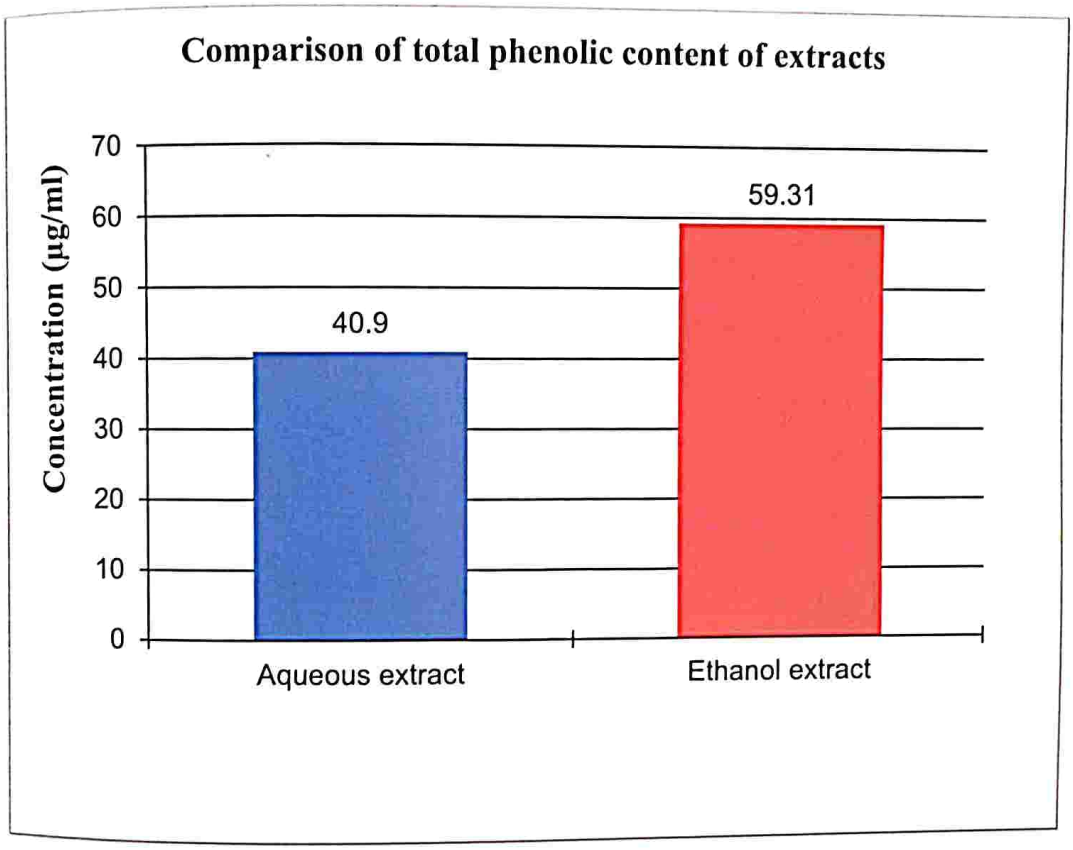


Fig No. 15: Comparison of the Total Phenolic content of Aqueous and ethanolic extract of *Mussaenda frondosa* Linn.

5.4.6. Thin layer chromatography

Table no. 19 : Thin layer chromatography of various extracts of *Mussaenda frondosa* Linn.

Sl.no	extracts	Solvent system	No. of spots
1.	Petroleum ether	Toluene : chloroform: acetone: formic acid (5:3:2:1drop)	3
2.	Chloroform	Toluene : chloroform: acetone: formic acid (5:3:2:1drop)	10
3.	Ethyl acetate	Toluene: Ethyl acetate: Acetonitrile (5:2:2)	3
4.	Ethanol	N-Hexane: Toluene: Ethyl acetate: Formic acid (3:5:3:0.6)	5
5.	Water	Ethyl acetate : Ethanol (2:1.5)	1

5.4.7. Column Chromatography

Column chromatography of ethanolic stem extract of *Mussaenda frondosa* Linn was done using isocratic elution technique using N-hexane: Toluene: Ethyl acetate: Formic acid (3:5:3:0.6)ml . 37 fractions of each 10 ml were collected. Thin layer chromatography was done for all the fractions and Rf values were found out. The fractions having similar Rf values were pooled together. Then, physical evaluation and preliminary phytochemical screening was conducted for all the pooled fractions

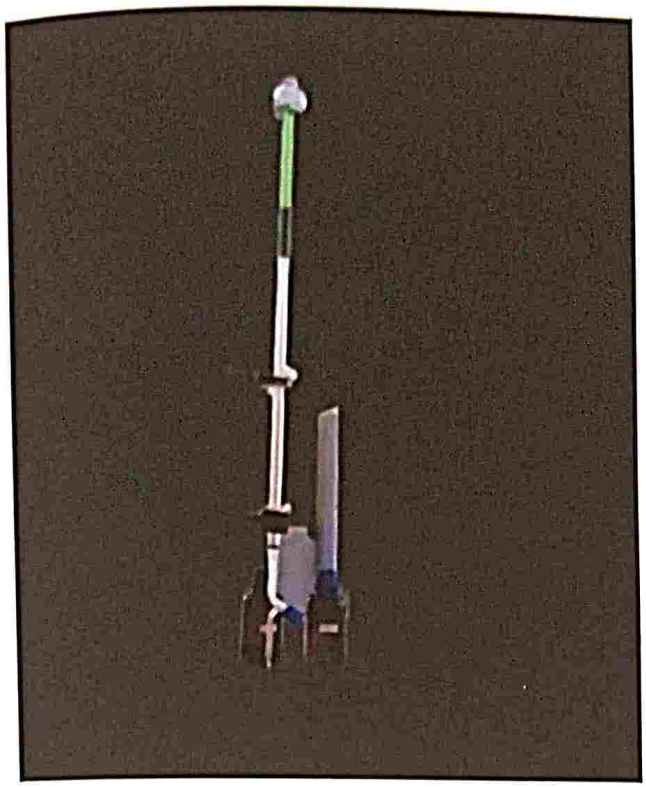


Fig No. 16: Column chromatography of Ethanolic extract of *Mussaenda frondosa* Linn.

Table no. 20 : Column chromatography of Ethanolic stem extract of *Mussaenda frondosa* Linn.

Sl. No.	Solvent system	Fraction	No of Spot	Colour of spot in UV	Rf value	Pooled
1	N-hexane: Toluene: Ethyl acetate: Formic acid (3:5:3:0.6)	F1	-	-	-	-
		F2				
2		F3	1	White (L)	0.68	A
		F4				
		F5				
		F6				
		F7				
3		F8	2	White (L)	0.68	B
		F9		Blue(S)	0.46	
4		F10	4	White (L)	0.68	C
				Blue(S)	0.46	
					0.37	

					0.33	
5		F11	1	White (L)	0.26	D
		F12				
6		F13	2	White (L)	0.26	E
					0.7	
7		F14	2	White(L)	0.42	F
		F15				
		F16		Blue(S)	0.31	
8		F17	-	Nil	-	
		F18				
		F19				
		F20				
		F21				
		F22				
9		F23	2	White (L)	0.2	G
		F24				
		F25				
		F26				
		F27			0.1	
		F28				
		F29				
		F30				
		F31				
10		F32	-	-	-	-
		F33				
		F34				
		F35				
		F36				
		F37				

L- Long wavelength Ultra Violet

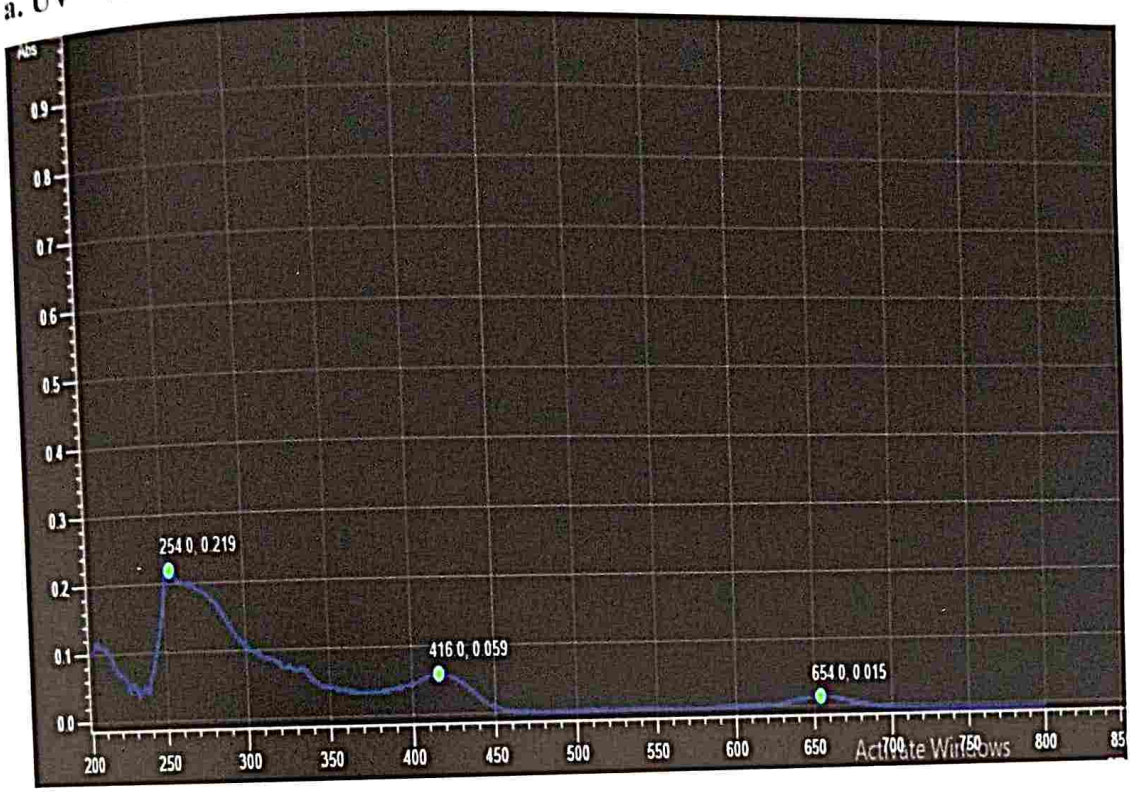
S- Short wavelength Ultra Violet

Fractions 3-7 and 11-12 had showed single spot with Rf (**retardation factor**) value 0.68 & 0.26 respectively .These fractions were pooled together according to their

similar Rf value and labelled as A and D respectively and Fraction A used for further spectral studies.

5.4.8. Spectral Studies

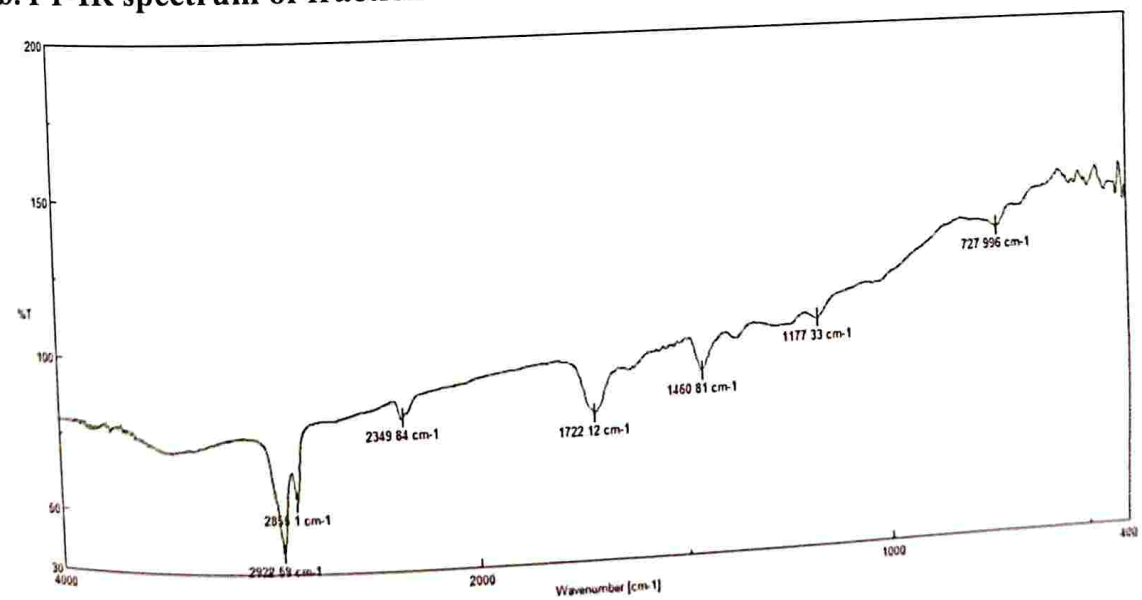
a. UV - Visible spectrum of fraction



Spectrum No. 1 : UV-Vis spectrum of isolated fraction A

Absorbance – 254nm, 416nm, 654 nm

b. FT-IR spectrum of fraction



Spectrum No.2 : FT-IR spectrum of isolated fraction A

Table No. 21:Interpretation of FT-IR spectrum

Sl no	Peak Value cm-1	Spectroscopic Assignments	Functional Groups
1.	2922	C-H stretch,	Alkane
		O-H stretch	Alcohol/ carboxylic acid,
2.	2855	C-H stretch,	Alkane,
		O-H stretch,	Alcohol/ carboxylic acid
		N-H stretch	Amine salt
3.	2349	O=C=O stretch	Carbon dioxide
4.	1722	C=O stretch	Aldehyde/ α,β-unsaturated ester/ aliphatic ketone
5.	1460	C-H bend	Alkane
6.	1177	C-O stretch	Ester/ tertiary alcohol
		C-F stretch	Fluoro compound
7.	727	C-H bend	1,2-disubstituted/ monosubstituted
		C=C bend	alkene

C) Compound identification via HR- LCMS

The crude ethanolic extract and fraction A collected from ethanolic extract of *Mussaenda Frondosa* Linn by coloum chromatography was subject to HR-LCMS

Table no.22: Phytoconstituents identified from HR-LCMS of ethanolic crude extract of *Mussaenda Frondosa* Linn

Sl no	Name	Formula	Molecular Weight	RT [min]	Remarks
1.	Y-Aminobutyric acid (GABA)	C4 H9 N O2	103.0633	0.88	PM, plays an important role in plant growth and abiotic stress mitigation in plants.
2.	DL-Carnitine	C7 H15 N O3	161.1052	0.98	PM

3.	Nicotinic acid	C6 H5 N O2	123.032	1.17	Vitamin B3
4.	Chlorogenic acid	C16 H18 O9	354.0951	8.47	PC, Ester of caffeic acid and quinic acid
5.	Caffeic acid	C9 H8 O4	180.0423	7.91	PC, Iintermediate in the biosynthesis of lignin
6.	Syringic acid	C9 H10 O5	198.05	6.32	PM, PC, Derivative of gallic acid.

PM- Plant Metabolites
PC- Phenolic Compound
RT- Retention time

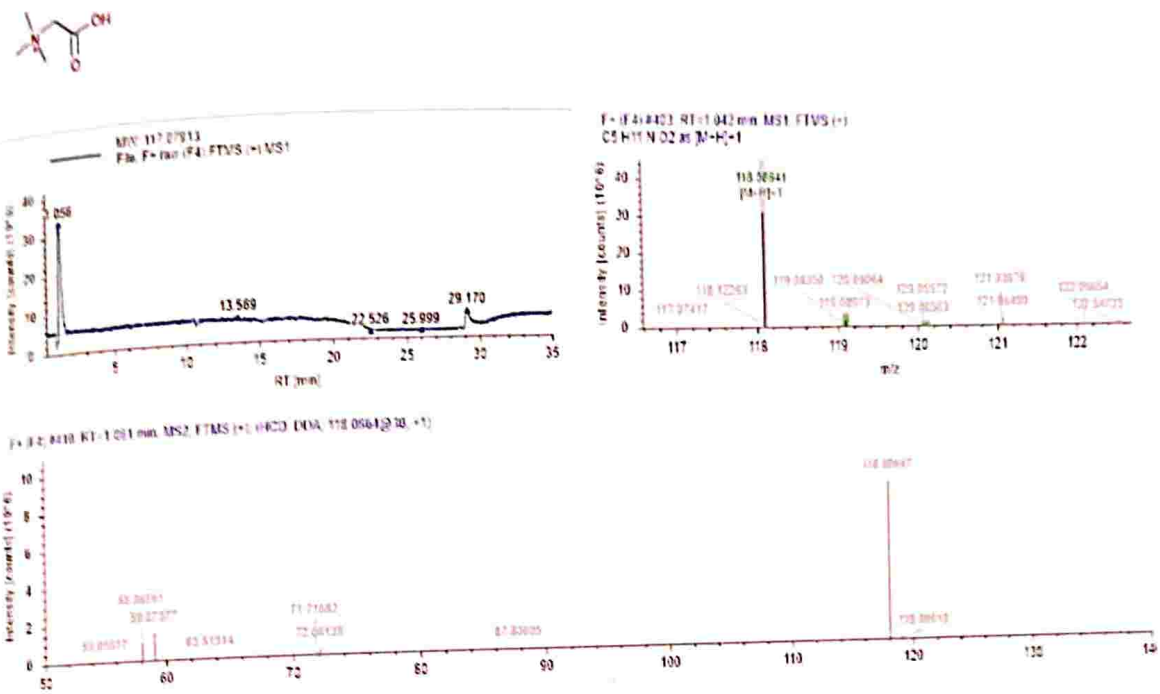
Table no.23: Phytoconstituents identified from HR-LCMS of crude ethanolic extract and fraction A collected from ethanolic crude extract of *Mussaenda Frondosa* Linn

Sl no	Name	Formula	Molecular Weight	RT(min)	Remarks
1.	Betaine	C5 H11 N O2	117.79	1.05	PM
2.	9-Oxo-10(E),12(E)-octadecadienoic acid	C18 H30 O3	294.21	18.38	Derived from tomato
3.	DL-Stachydrine	C7 H13 N O2	143.09	1.15	Alkaloid
4.	Hexadecanamide	C16 H33 N O	255.25	21.73	Fatty amide that is the carboxamide derived from Palmitic Acid
5.	L-Glutamic acid	C5 H9 N O4	147.05	0.96	Amino Acid, Plays a

					very important role in plant growth and development.
6.	NP-000294	C15 H22 O2	234.16	17.71	-
7.	16-Hydroxyhexadecanoic acid (Juniperic acid)	C16 H32 O3	272.23	20.99	Omega-hydroxy long-chain fatty acid that is palmitic acid which is substituted at position 16 by a hydroxy group
8.	(±)9-HpODE (hydroperoxy-octadecadienoic acid)	C18 H32 O4	312.22	16.30	Racemic mixture of fatty acid
9.	Arecoline	C8 H13 N O2	155.09	1.25	Alkalolid
10.	D-Raffinose	C20 H30 N4 O14	550.17	0.89	Carbohydrate, trisaccharide composed of galactose, glucose, and fructose

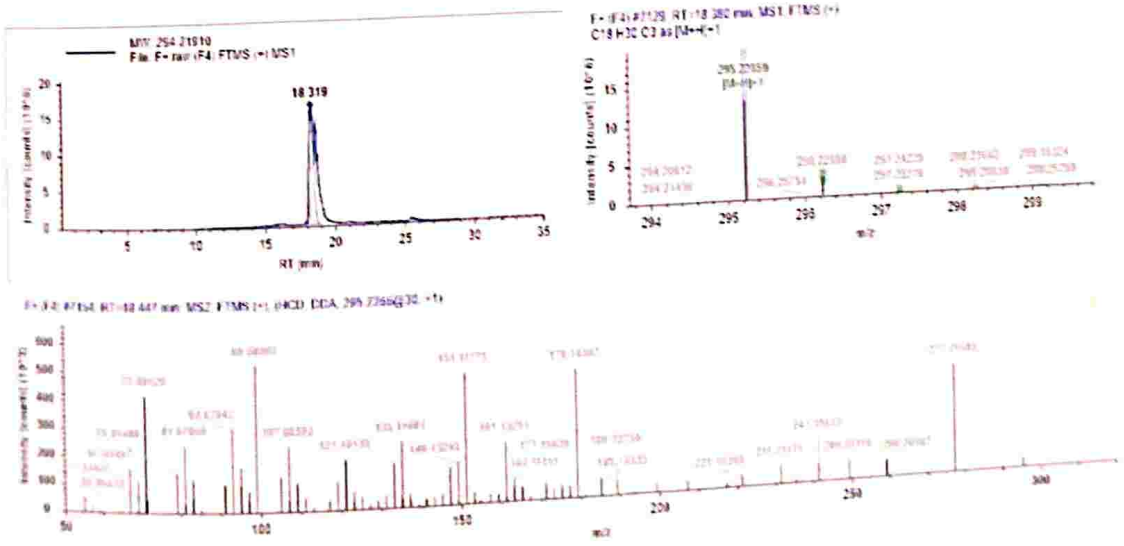
Spectrum No. 3 : HR- LCMS spectrum of phytoconstituents seen in both isolated fraction A and crude ethanolic extract of *Mussaenda frondosa* Linn.

Structure	Name	RT [min]	Formula	Molecular Weight	Group Areas
	Betaine	1.05	C5 H11 N O2	n/a	n/a



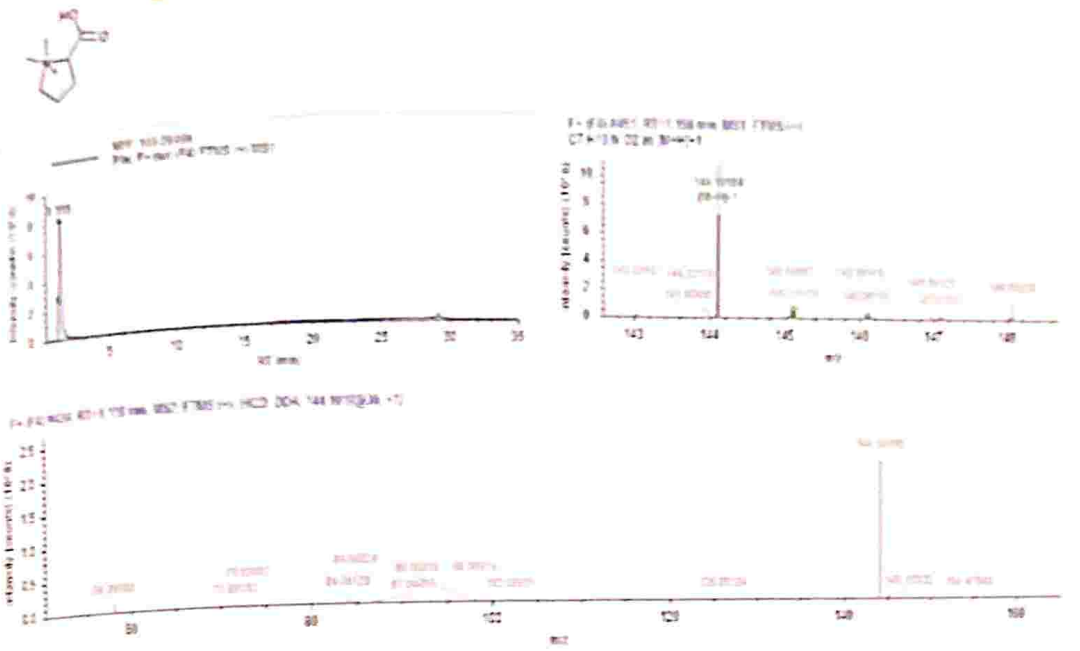
Spectrum: 3(i): Betaine

Structure	Name	RT [min]	Formula	Molecular Weight	Group Areas
	9-Oxo-10(E),12(E)-octadecadienoic acid	18.38	C18 H30 O3	n/a	n/a



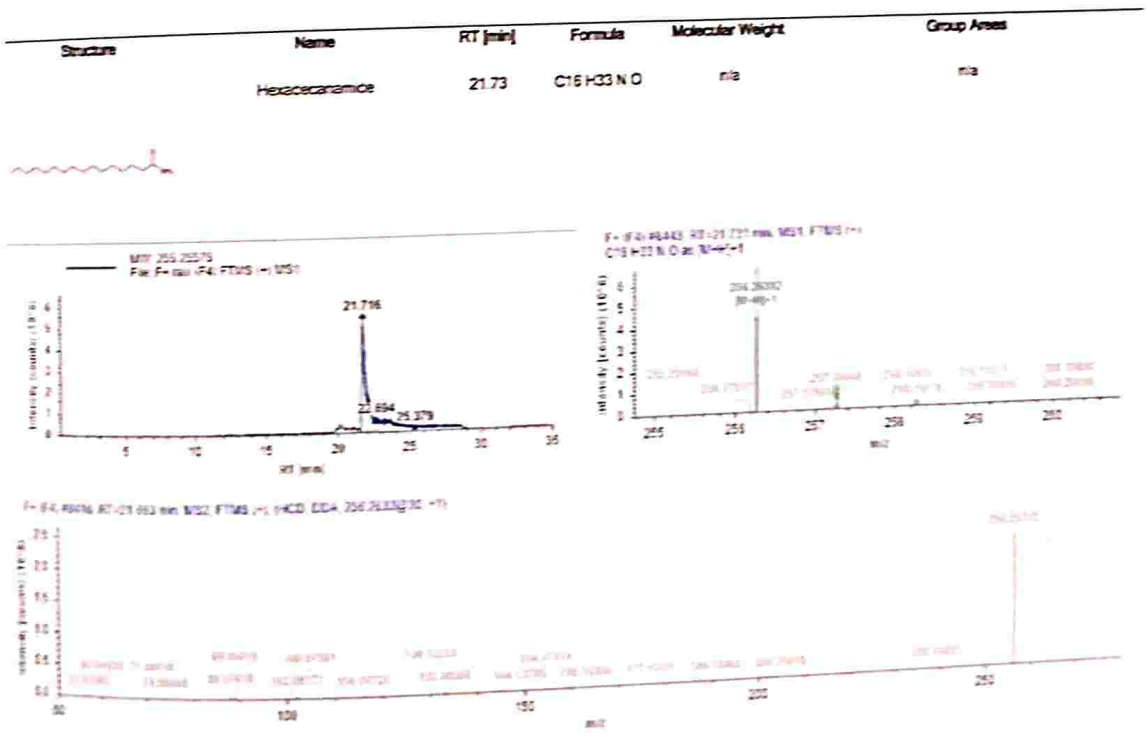
Spectrum. 3(ii); 9-Oxo-10(E),12(E)-octadecadienoic acid

Structure	Name	RT [min]	Formula	Molecular Weight	Group Areas
	DL-Stachydrine	1.15	C7 H13 N O2	n/a	n/a



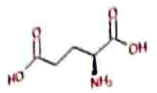
Spectrum. 3(iii):DL-Stachydrine

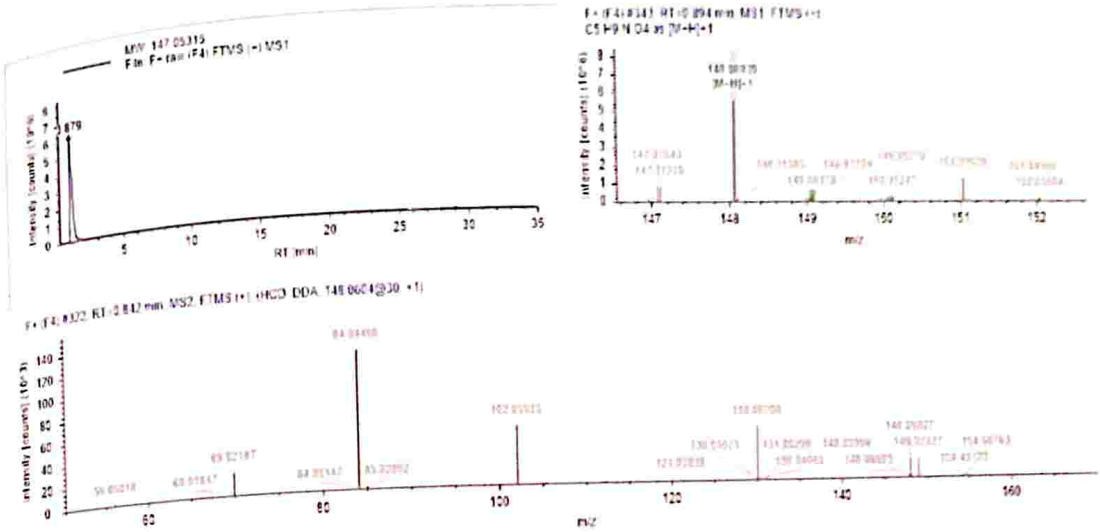
Structure	Name	RT [min]	Formula	Molecular Weight	Group Areas
	Hexadecanamide	21.73	C16 H33 N O	n/a	n/a



Spectrum. 3(iv): Hexadecanamide

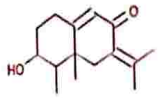
Structure	Name	RT [min]	Formula	Molecular Weight	Group Areas
	L-Glutamic acid	0.96	C5 H9 N O4	n/a	n/a

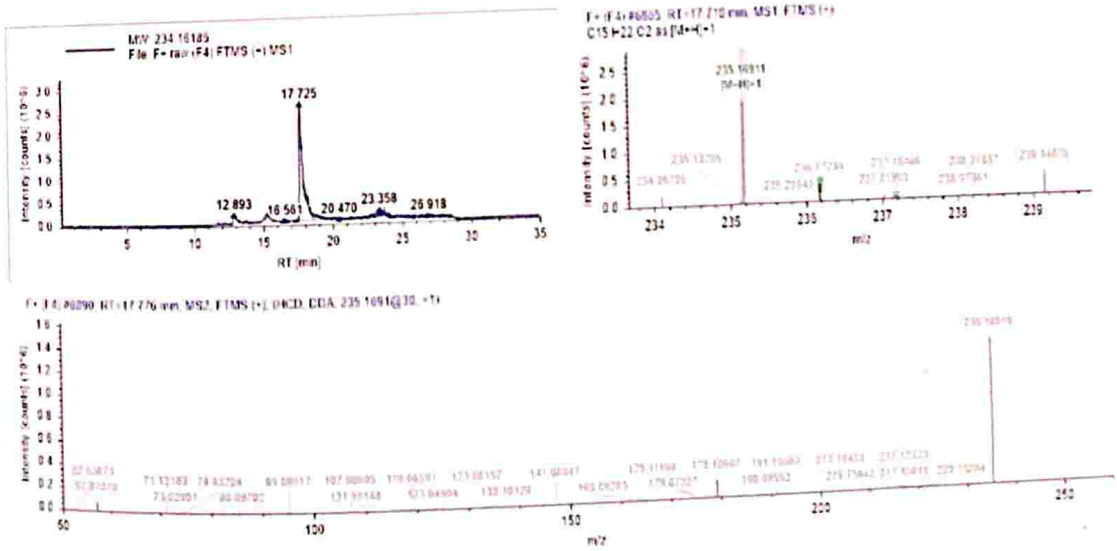




Spectrum. 3(v): L-Glutamic acid

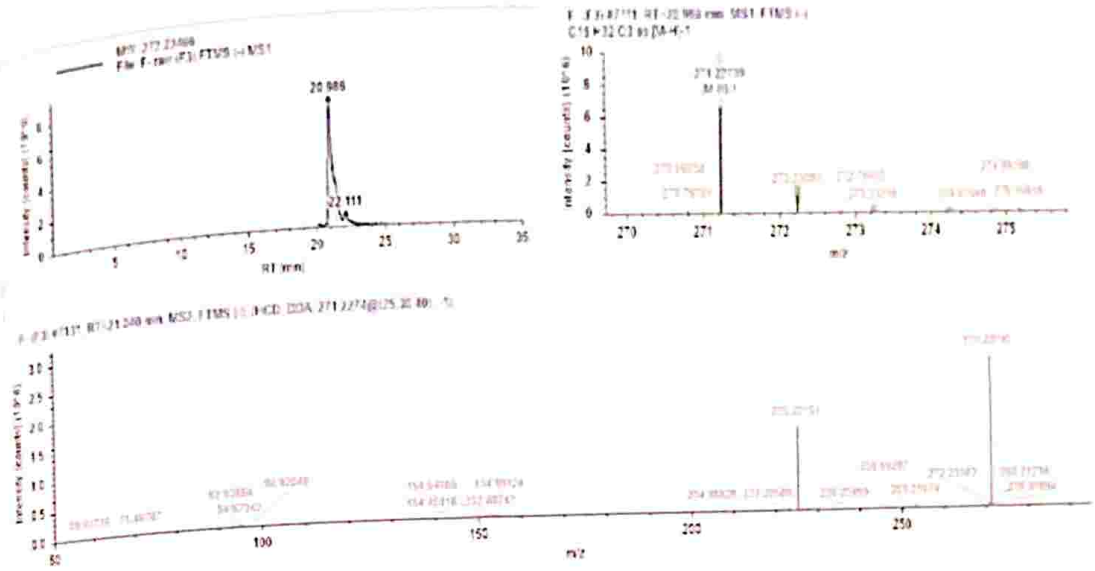
Structure	Name	RT [min]	Formula	Molecular Weight	Group Areas
	NP-000294	17.71	C15 H22 O2	n/a	n/a





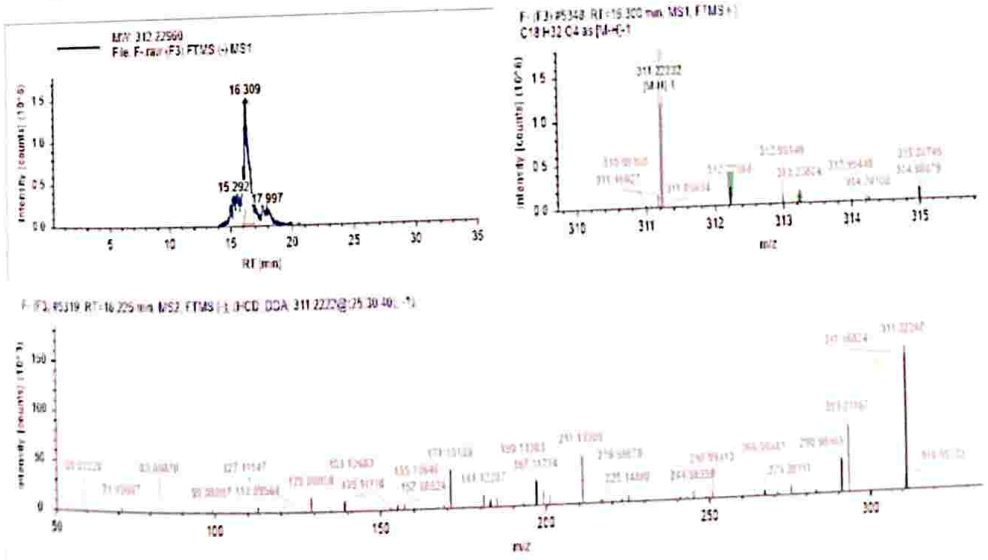
Spectrum. 3(vi):NP-000294

Structure	Name	RT [min]	Formula	Molecular Weight	Group Areas
	16-Hydroxyhexadecanoic acid	20.99	C16 H32 O3	n/a	n/a

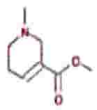


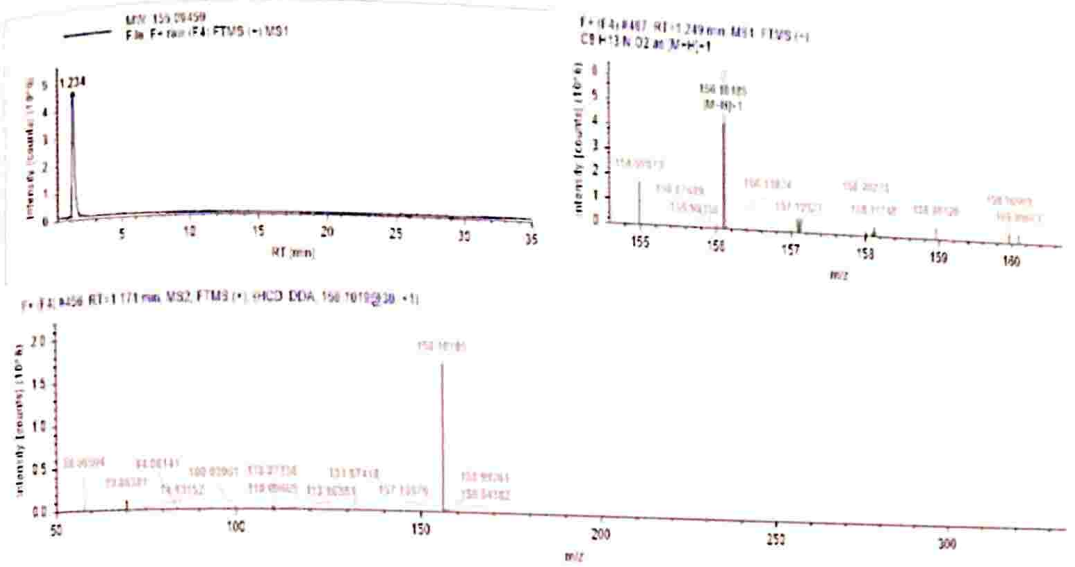
Spectrum. 3(vii): 16-Hydroxyhexadecanoic acid (Juniperic acid)

Structure	Name	RT [min]	Formula	Molecular Weight	Group Areas
	(±)9-HpODE	16.30	C18 H32 O4	n/a	n/a



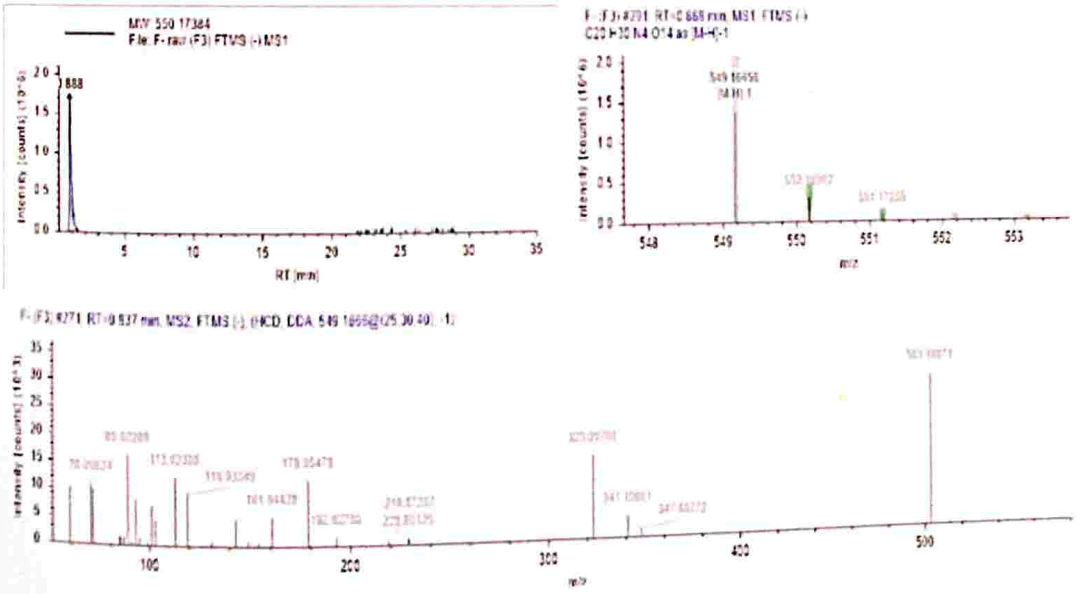
Spectrum. 3(viii): (±)9-HpODE (hydroperoxy-octadecadienoic acid)

Structure	Name	RT [min]	Formula	Molecular Weight	Group Areas
	Arecoline	1.25	C8 H13 N O2	n/a	n/a



Spectrum. 3(ix): Arecolin

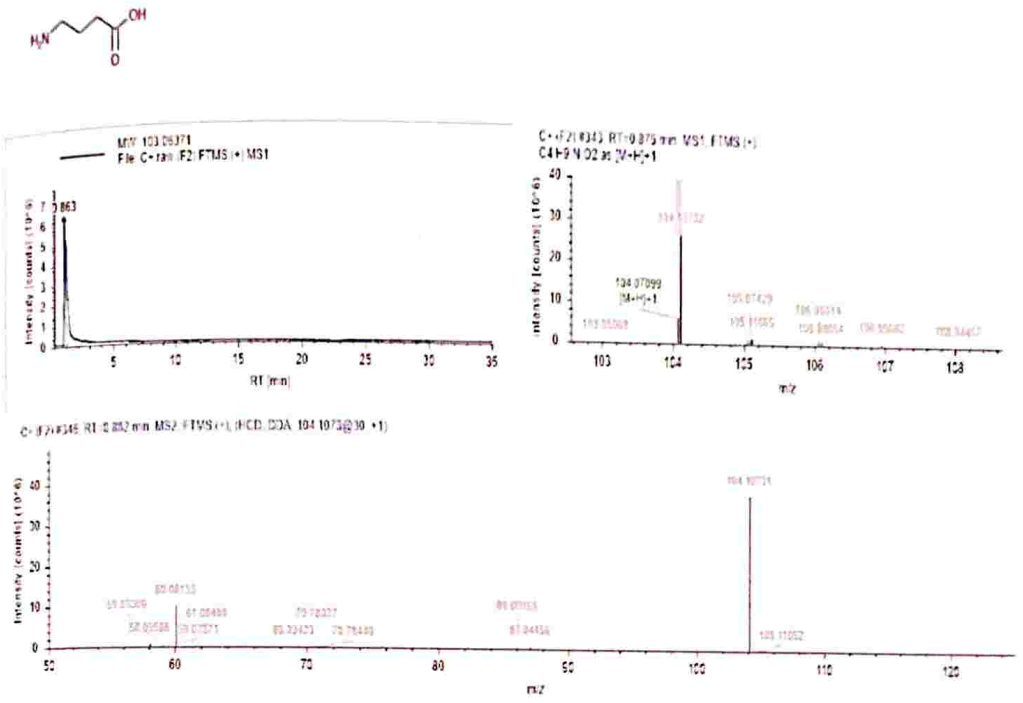
Structure	Name	RT [min]	Formula	Molecular Weight	Group Areas
	[Similar to: D-Raffinose; ΔMass: 46.0048 Da]	0.89	C20 H30 N4 O14	n/a	n/a



Spectrum. 3(x): D- Raffinose

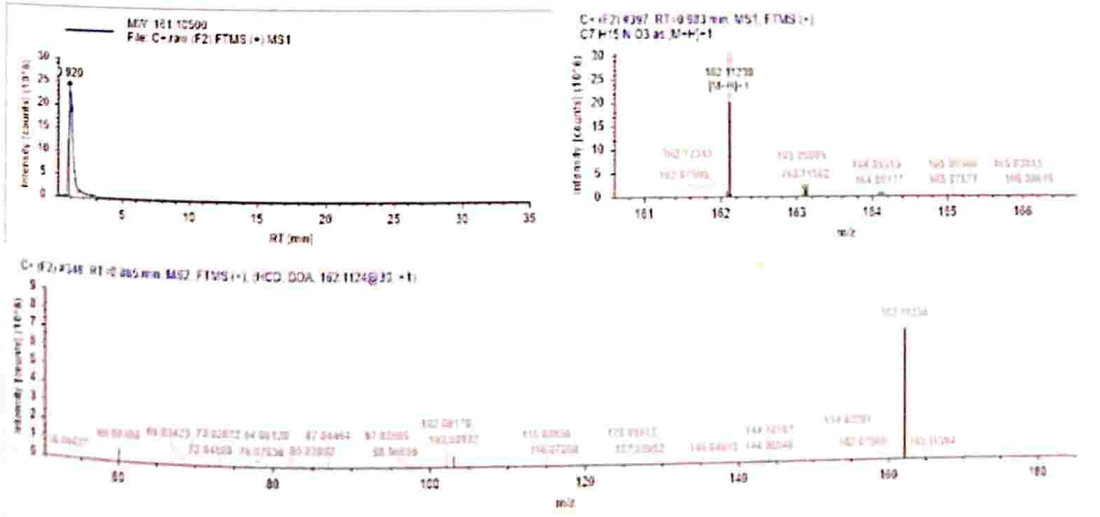
Spectrum No. 4 : HR- LCMS spectrum of phytoconstituents seen only in crude ethanolic extract of *Mussaenda frondosa* Linn.

Structure	Name	RT [min]	Formula	Molecular Weight	Group Areas
	Y-Aminobutyric acid (GABA)	0.88	C4 H9 N O2	n/a	n/a



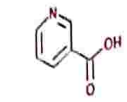
Spectrum. 4(i): Y-Aminobutyric acid (GABA)

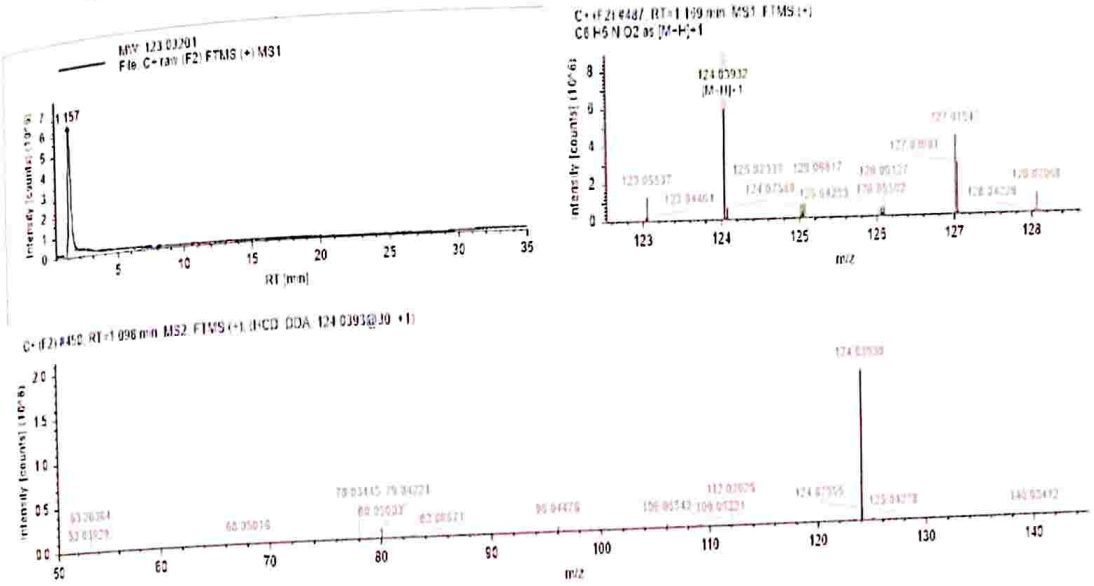
Structure	Name	RT [min]	Formula	Molecular Weight	Group Areas
	DL-Carnitine	0.98	C7 H15 N O3	n/a	n/a



Spectrum. 4(ii):DL-Carnitine

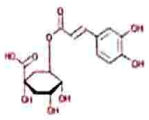
Structure	Name	RT [min]	Formula	Molecular Weight	Group Areas
	Nicotinic acid	1.17	C6 H5 N O2	n/a	n/a

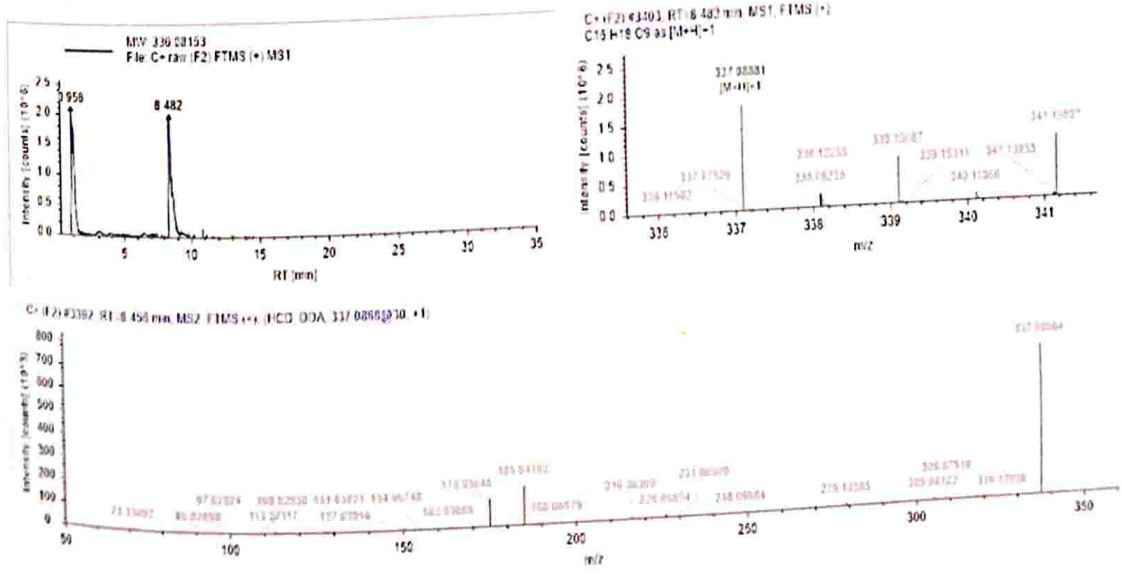




Spectrum. 4(iii): Nicotinic acid

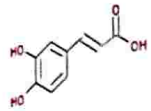
Structure	Name	RT [min]	Formula	Molecular Weight	Group Areas
	Chlorogenic acid	8.47	C16 H18 O9	n/a	n/a

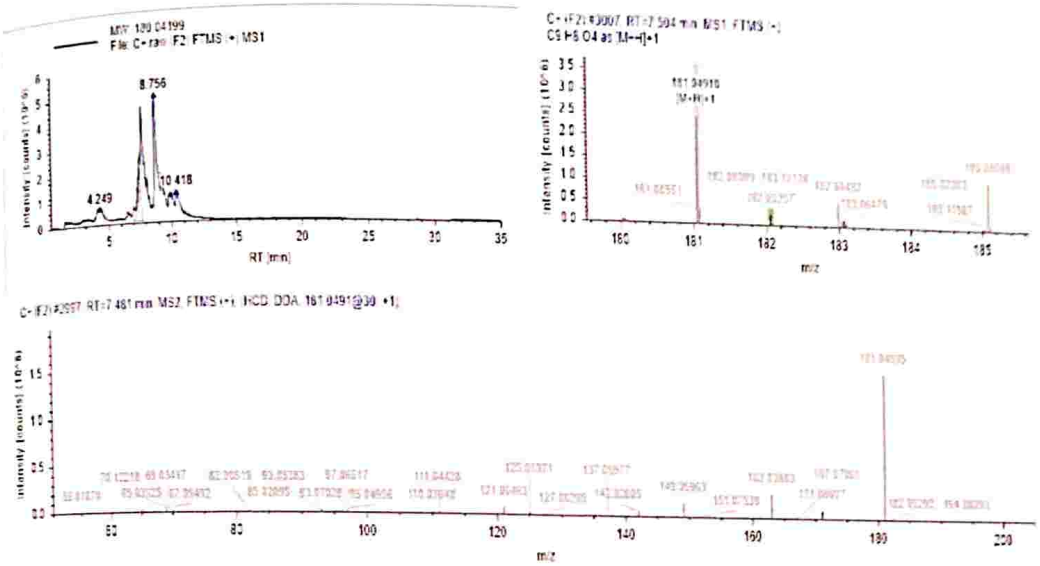




Spectrum. 4(iv): Chlorogenic acid

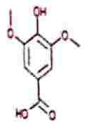
Structure	Name	RT [min]	Formula	Molecular Weight	Group Areas
	Caffeic acid	7.50	C9 H8 O4	n/a	n/a

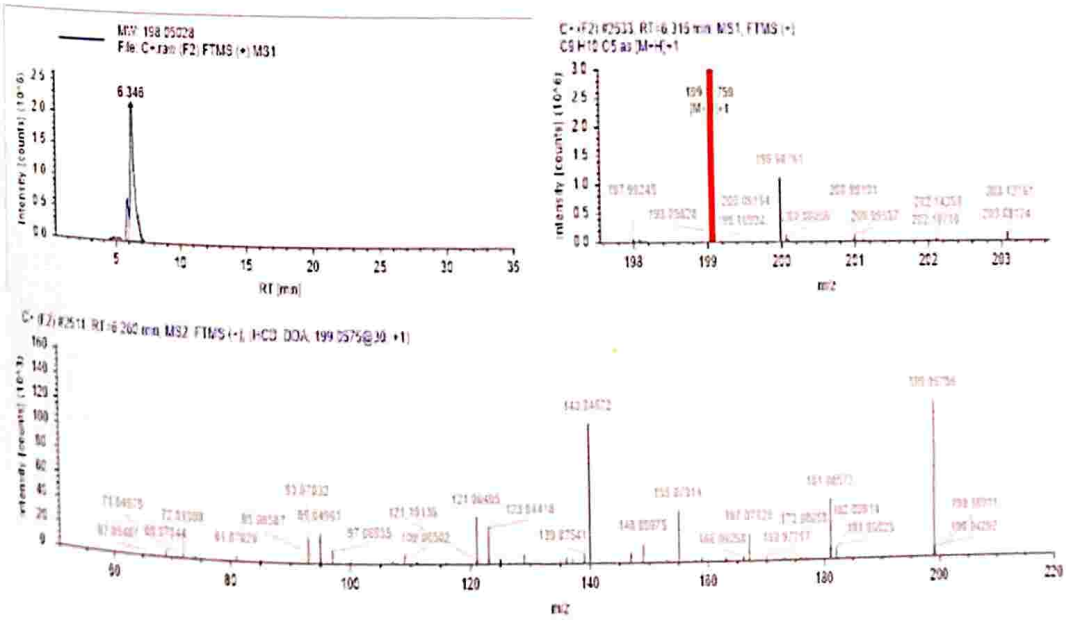




Spectrum. 4(v): Caffeic acid

Structure	Name	RT [min]	Formula	Molecular Weight	Group Areas
	Syringic acid	6.32	C9 H10 O5	n/a	n/a





Spectrum. 4(vi):Syringic acid

5.5. IN-VITRO PHARMACOLOGICAL STUDIES

5.5.1. ANTI-OXIDANT ACTIVITY

A. DPPH (2, 2- DIPHENYL- 1- PICRYL HYDRAZYL) ASSAY

The percentage inhibitions obtained in the different concentrations of extracts and percentage inhibition obtained with the standard were analysed and it is depicted below.

Table no. 24: Mean absorbance and percentage inhibition of standard Ascorbic acid

Sl. No.	Sample	Concentration (µg/ml)	Mean absorbance	Percentage Inhibition
1.	Control	-	0.683±0.0003	-
2.	Standard Ascorbic acid	5	0.426±0.002	37.62
		10	0.398±0.003	41.73
		15	0.304±0.004	55.49
		20	0.17±0.002	75.1
		25	0.071±0.0043	89.60

Values are expressed in mean ± SEM, n = 3, a= $p<0.0001$ compare to control and hence considered significant

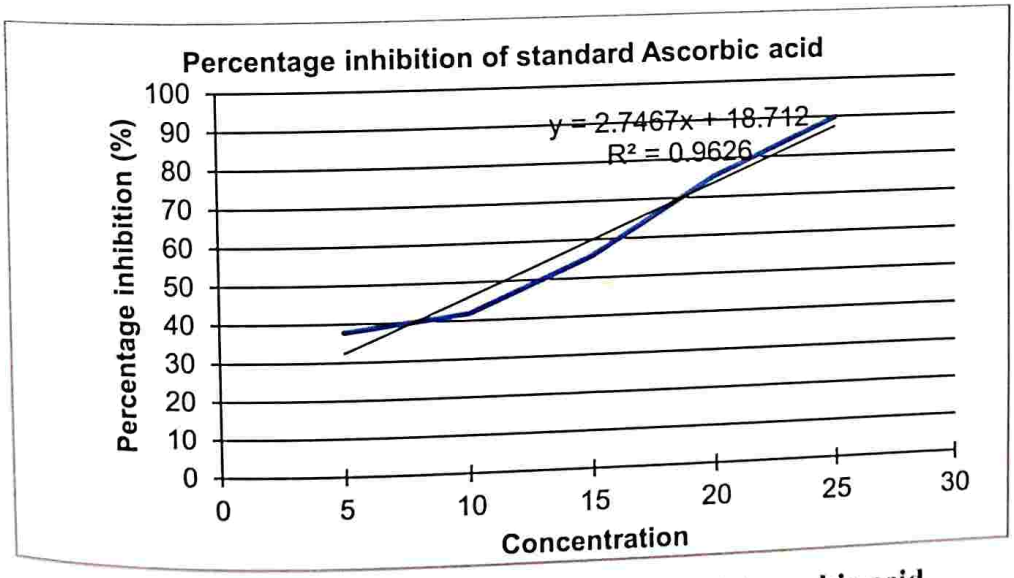


Fig No. 17: Percentage inhibition of standard Ascorbic acid

Table No. 25: Percentage inhibition of various extracts of *Mussaenda frondosa* Linn.

Sample	Concentration (µg/ml)	Absorbance at 517 nm	Percentage inhibition
Control	-	0.81±0.002	-
Petroleum ether	100	0.7985±0.0015	1.42
	200	0.789±0.01	2.59
	300	0.7705±0.0005	4.88
	400	0.761±0.004	6.05
Control		1.008±0.0051	
Chloroform	100	0.8505±0.0185	15.62
	200	0.8025±0.0075	20.39
	300	0.7215±0.0045	28.42
	400	0.6675±0.0055	33.78
Control		0.676±0.003	
Ethyl acetate	100	0.5045±0.0345	25.37
	200	0.4634±0.0025	31.43
	300	0.354±0.008	47.63
	400	0.264±0.005	60.95
Control		0.803±0.002	
ethanol	100	0.636±0.014	20.80
	200	0.54±0.001	32.75
	300	0.405±0.004	49.56
	400	0.2875±0.0115	64.20
Control		0.784±0.007	
Water	100	0.639±0.002	18.49
	200	0.55±0.005	29.85
	300	0.4505±0.0055	42.54
	400	0.3735±0.0005	52.36

Values are expressed in mean ± SEM, n = 3, a= $p<0.0001$ compare to control and hence considered significant

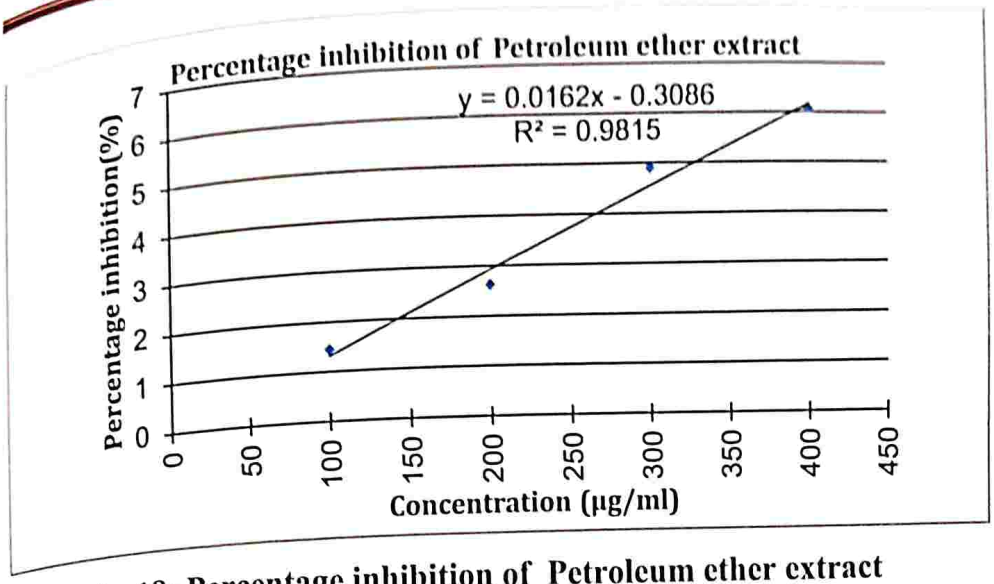


Fig No. 18: Percentage inhibition of Petroleum ether extract

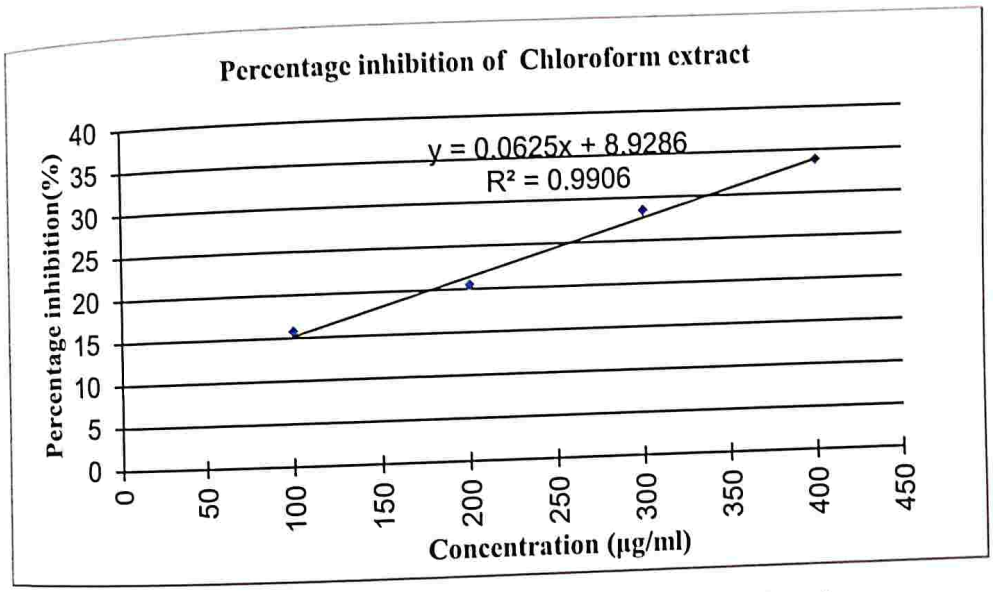


Fig No. 19: Percentage inhibition of Chloroform extract

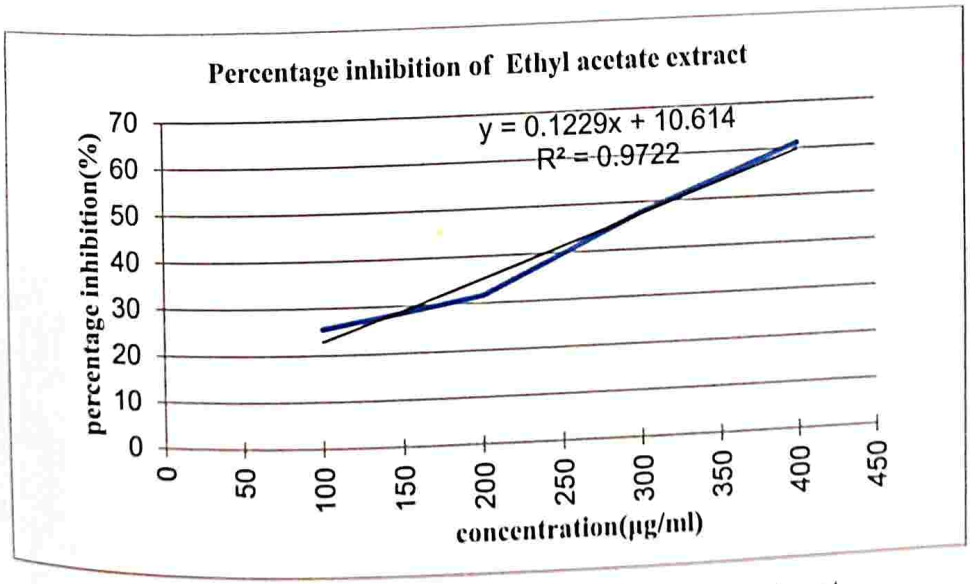


Fig No. 20: Percentage inhibition of Ethyl acetate extract

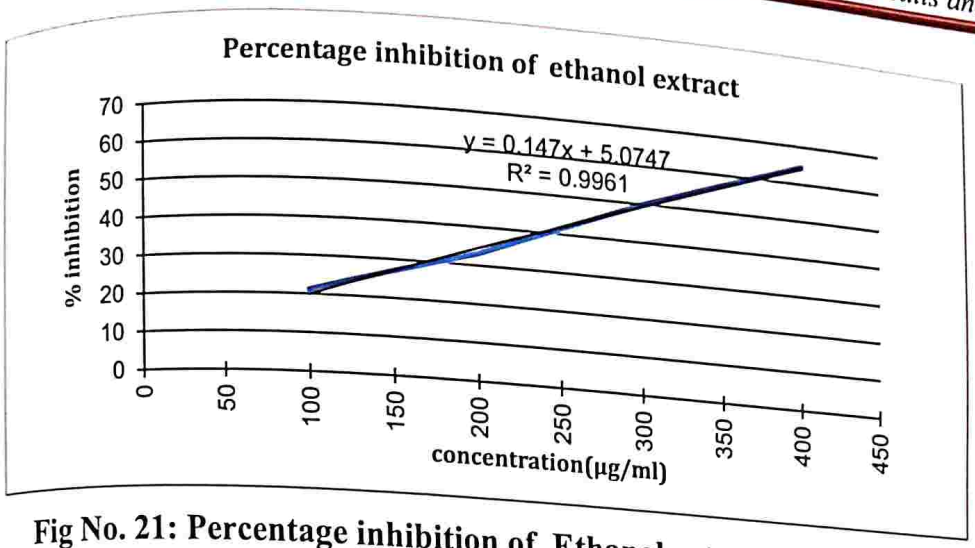


Fig No. 21: Percentage inhibition of Ethanol extract

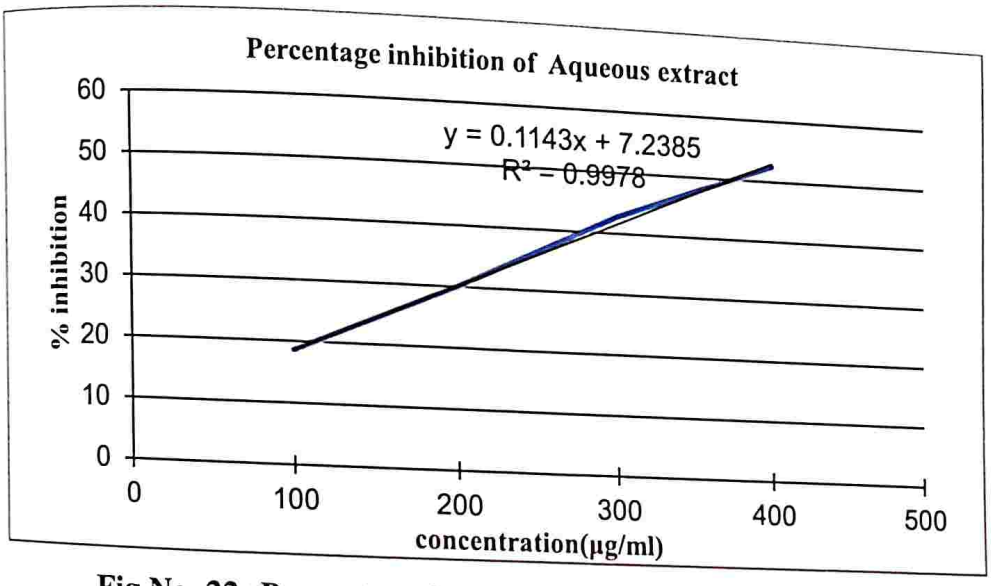


Fig No. 22: Percentage inhibition of water extract

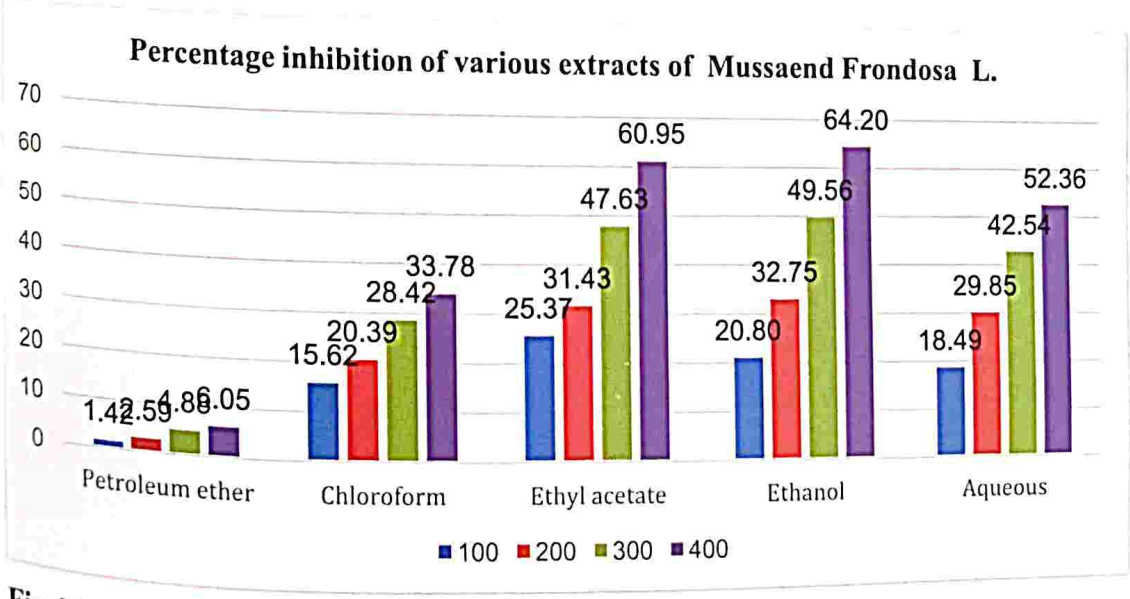


Fig No. 23: Percentage inhibition of various extract of *Mussaenda frondosa* Linn.

Table no. 26: IC $_{50}$ value of standard Ascorbic acid and various extracts of *Mussaenda frondosa* Linn.

Sl. No.:	Sample	IC_{50} value (µg/ml)
1.	Standard ascorbic acid	11.39
2.	Petroleum ether	3105.46
3.	Chloroform	657.142
4.	Ethyl acetate	320
5.	Ethanol	305.59
6.	Water	374.14

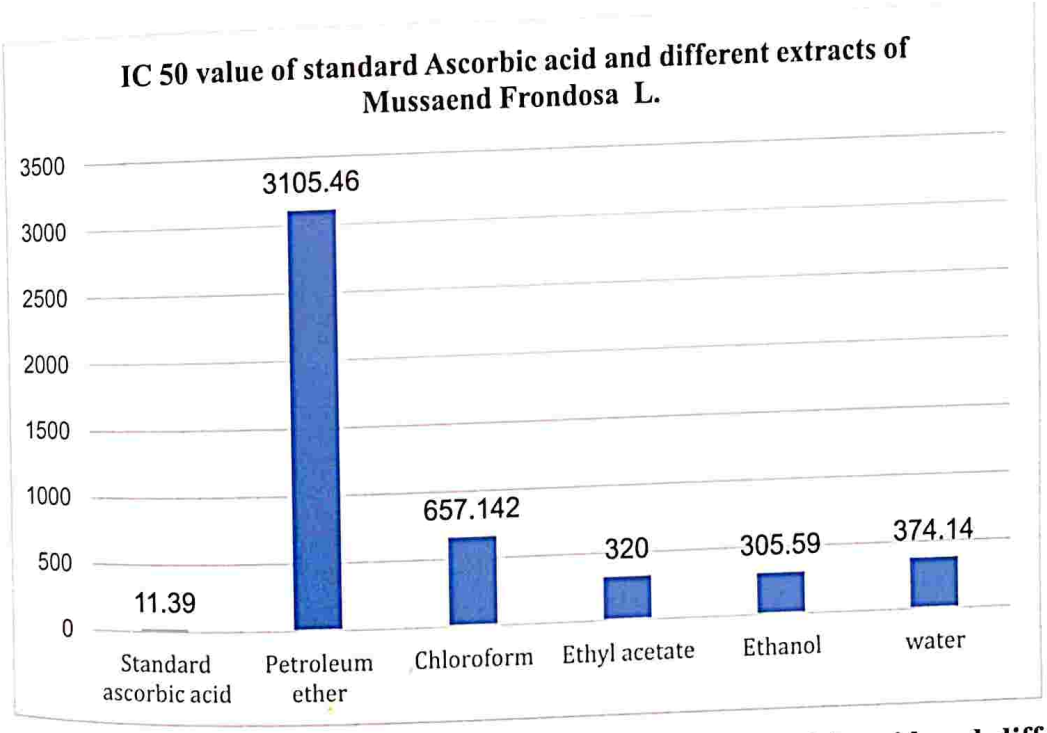


Fig No. 24: Comparison of IC $_{50}$ value of standard Ascorbic acid and different extracts of *Mussaenda frondosa* Linn.

Ethanolic extract of *Mussaenda frondosa* Linn had most percentage of inhibition and least IC50 value compare to other extracts, hence ethanolic extract selected for further studies.

5.5.3. ANTI-INFLAMMATORY ACTIVITY

A- Protein Denaturation Method

Table no. 27: Absorbance and mean percentage inhibition of Ethanolic stem extract of *Mussaenda frondosa* Linn. and standard drug Diclofenac Sodium.

Diclofenac sodium Standard			
Sl no		**Mean Optical density**	**Percentage Inhibition**
	Control	0.1069±0.037	-
1	62.5	0.0793 ±0.045	25.77
2	125	0.047±0.015	55.14
3	250	0.0230±0.017	78.48
4	500	0.0113±0.0059	89.39

Values are expressed in mean ± SEM, n = 3,

Ethanolic extract			
Sl no		**Mean optical density**	**Percentage Inhibition**
	Control	0.1069±0.037	-
1.	62.5	0.0960±0.004	10.11
2.	125	0.0790±0.0048	26.04
3.	250	0.0473±0.0054	55.69
4.	500	0.0366±0.0075	65.76

Values are expressed in mean ± SEM, n = 3,

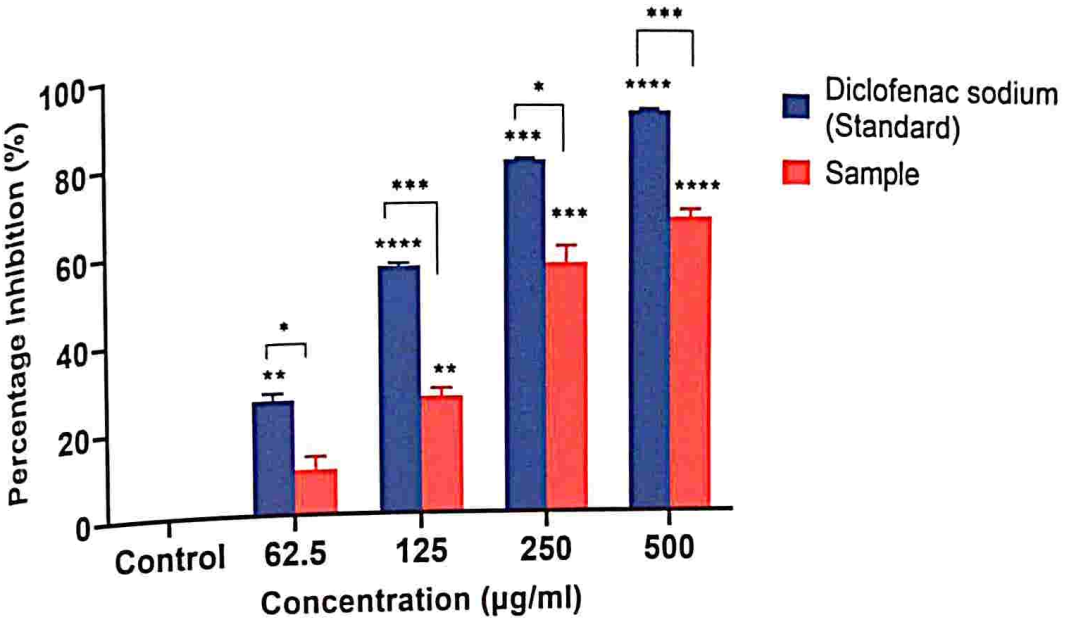


Figure: Graphical representation depicting the protein denaturation activity of Diclofenac sodium Standard and test drug. Along Y axis Percentage inhibition, Along X axis varied concentration of Diclofenac sodium Standard and test drug. All experiments were done in triplicates and results represented as Mean+/- SE. One-way ANOVA and *Tukey*'s test were performed to analyse data. ***p< 0.001 compared to control group.

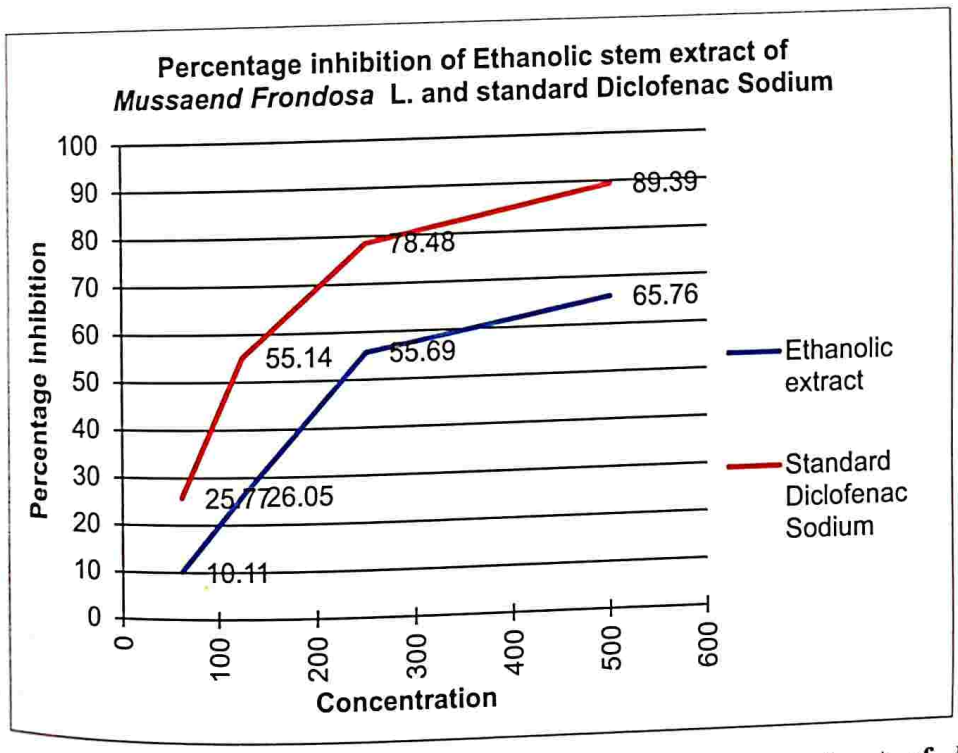


Fig No. 25: Percentage inhibition of Ethanolic stem extract of *Mussaenda frondosa* Linn. and standard Diclofenac Sodium.

Table no. 28: IC $_{50}$ values of standard Diclofenac Sodium and Ethanolic stem extract of *Mussaenda frondosa* Linn.

Sl. No:	Sample	IC $_{50}$ value (µg/ml)
1.	Standard Diclofenac Sodium	140
2.	Ethanolic stem extract	321.02

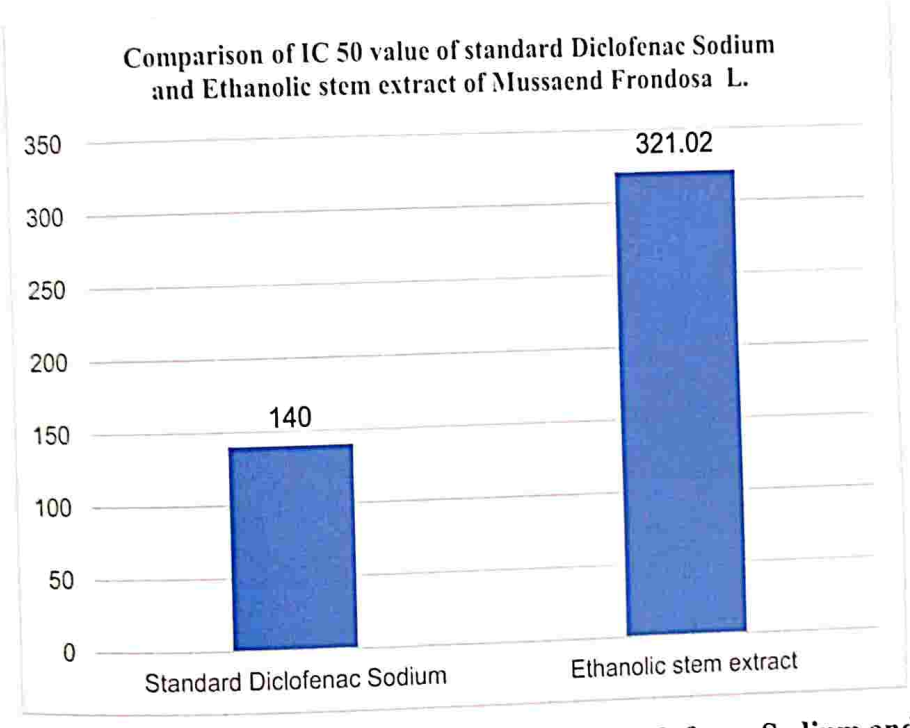


Fig No. 26: Comparison of IC $_{50}$ value of standard Diclofenac Sodium and Ethanolic stem extract of *Mussaenda frondosa* Linn.

Ethanolic extract of *Mussaenda frondosa* Linn has mild anti-inflammatory activity compare to standard Diclofenac Sodium

5.5.5. ANTI-MICROBIAL ACTIVITY

a. ANTI-BACTERIAL ACTIVITY

Positive control: Standard Streptomycin

Negative control: DMSO

Organism: *Staphylococcus aureus.*

Table no. 29: Comparison of the zone of inhibition formed by the effect of Ethanolic stem extract of *Mussaenda frondosa* Linn. against standard Streptomycin

Sample	Concentration (µg)	Mean Zone of inhibition(mm)
Control	-	-
Standard Streptomycin	100	27
Ethanolic extract	250	Nil
	500	Nil
	1000	Nil

Values are expressed in mean, n = 2,

Fig No. 27: Anti-bacterial activity of Ethanolic stem extract of *Mussaenda frondosa* Linn. against *Staphylococcus aureus.*

Ethanolic extract of *Mussaenda frondosa* Linn showed no anti-microbial activity compare to standard Streptomycin

b. ANTI - FUNGAL ACTIVITY

Positive control: Clotrimazole

Negative control: DMSO

Organism: *Candida albicans*

Table no. 30: Comparison of the zone of inhibition formed by the effect of Ethanolic stem extract of *Mussaenda frondosa* Linn against standard Clotrimazole

Sample	Concentration (µg)	Mean Zone of inhibition (mm)
Control	-	-
Standard Clotrimazole	100	33.5
Ethanolic extract	250	Nil
	500	Nil
	1000	20

Values are expressed in mean, n = 2,

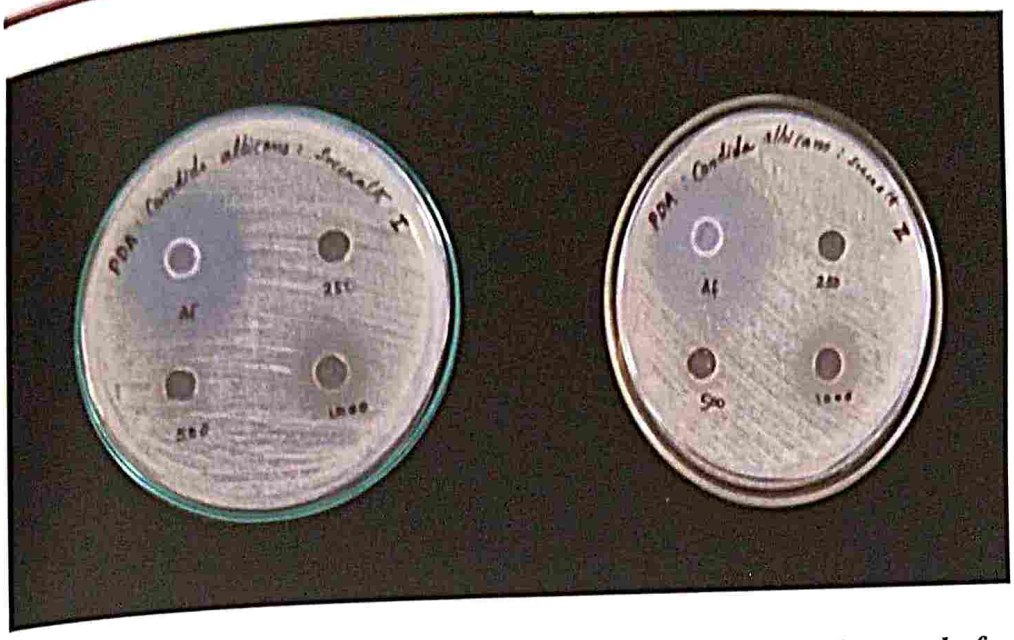


Fig No. 28: Anti-fungal activity of Ethanolic stem extract of *Mussaenda frondosa* Linn. against *Candida albicans*

Ethanolic extract of *Mussaenda frondosa* Linn showed mild anti-fungal activity compare to standard Clotrimazole

SUMMARY AND CONCLUSION

6. SUMMARY AND CONCLUSION

This thesis work deals with the Pharmacognostical, Phytochemical and *In vitro* Pharmacological studies on the stem of *Mussaenda frondosa* Linn belonging to the family, Rubiaceae.

Fresh stem were collected from Pachaloor, Thiruvananthapuram district. The collected stem were carefully examined and authenticated by the curator Dr. E.A. Siril, Proffessor and Head. , Department of Botany, University of Kerala, Kariavattom, Thiruvananthapuram. A voucher specimen (KUBH 11152) has been deposited for future reference.

The collected stem were washed, dried under shade and powdered.

Pharmacognostical studies were done on fresh stem and dried powdered stem which covered the macroscopical and microscopical studies. These microscopical and macroscopical features will help in the authentication of plant material.

The standardization of the drug was conducted by determination of various Physicochemical parameters like ash values, extractive values, loss on drying, detection of inorganic constituents and fluorescence behavior etc, and these parameters helps to develop standards and to determine purity and quality of the drug.

The coarse powder was used for Successive Maceration and Soxhlation using solvents such as Petroleum ether, Chloroform, Ethyl acetate, Ethanol and Chloroform water. Soxhlet extraction using aqueous solvent given the maximum yield.

Preliminary phytochemical screening was done on all the extracts to identify the presence of various primary and secondary metabolites. Petroleum ether extract contain steroids. Ethyl acetate and Chloroform extract contains steroid, carbohydrates, flavanoids and glycoside. Aqueous extract contains tannins. Carbohydrates, flavanoid, tannins were present on ethanolic extract. Quantitative determination of tannins, flavonoids and phenolics were done by standard colorimetric methods.

TLC of each extracts were conducted by trial and error methods and suitable solvent system was developed. Rf values of the separated spots were calculated.

Anti-oxidant activities of all the extracts were determined by DPPH assay using ascorbic acid as standard. Comparison of antioxidant activity of all the extracts revealed that ethanolic extract has the maximum antioxidant activity compare to other extracts. So the ethanolic extract was selected for further chromatographical and pharmacological studies.

Column chromatography was performed on the ethanolic extract by isocratic elution using N-hexane: Toluene: Ethyl acetate: Formic acid (3:5:3:0.6) as solvent. Total 37 fractions were collected. The fractions 3-7 and 11-12 showed single spot with same Rf value. They were pooled and labelled as A and D . The pooled fraction A was used for further spectral studies.

UV-Visible, FT-IR and HR-LCMS spectral studies are done. Chlorogenic acid, Caffeic acid, Syringic acid, 16-Hydroxyhexadecanoic acid (Juniperic acid), Arecoline, Hexadecanamide etc were identified by HR-LCMS.

In-vitro anti-oxidant activity of all the extracts were determined by DPPH and compared with standard ascorbic acid. Ethanolic extract shows significant anti-oxidant activity as compared to other extracts but very mild anti-oxidant activity as compared to standard.

Anti-inflammatory activity of ethanolic extract was determined by protein denaturation method (Bovine Serum Albumin) using Diclofenac sodium as standard. The ethanolic extract showed mild anti-inflammatory activity as compared to standard.

Antimicrobial study was done using *Staphylococcus aureus* and *Candida albicans*. The study showed no anti-bacterial activity against *Staphylococcus aureus* but had mild activity against *Candida albicans*.

Mussaenda frondosa Linn is used in Indian system of medicine and folk medicinal practices, so it is necessary to standardize as a usable drug. The primary steps for establishing the quality control profile of any plant drug is almost covered by all these studies. According to WHO standardization protocol, botanical standards should be proposed as a protocol for the diagnosis of the herbal drugs. All these studies on the stem of *Mussaenda frondosa Linn.* belonging to the family Rubiaceae can be utilized for the standardization of the drug. Further Pharmacological and Phytochemical studies are needed to verify the therapeutic activity and to isolate the

bioactive components which are responsible for the mild pharmacological activity. Further spectral studies are needed to yield promising drug candidate.

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8. ANNEXURE

8.1. LIST OF CHEMICALS, SOLVENTS AND REAGENTS

Sl No.	Chemicals / Reagents	Manufacturer
1.	Acetic anhydride	Merck
2.	Acetone	Merck
3.	Acetic acid	Central drug house Pvt Ltd
4.	Aluminium chloride	Central drug house Pvt Ltd
5.	Ammonium molybdate	Central drug house Pvt Ltd
6.	Ammonium oxalate	Central drug house Pvt Ltd
7.	Ammonia	Central drug house Pvt Ltd
8.	Barfoed's reagent	Central drug house Pvt Ltd
9.	Barium chloride	Central drug house Pvt Ltd
10.	Benedict's reagent	Universal Chemicals and scientific industries South India
11.	Biuret reagent	Kanton Laboratories
12.	Borntrager's reagent	Central drug house Pvt Ltd
13.	Bromine	Central drug house Pvt Ltd
14.	Carbon tetra chloride	Merck
15.	Chloral hydrate	Central drug house Pvt Ltd

16.	Chloroform	Merck
17.	Copper turnings	Central drug house Pvt Ltd
18.	Cyclohexane	Central drug house Pvt Ltd
19.	Di ethyl ether	Central drug house Pvt Ltd
20.	Dimethyl sulphoxide (DMSO)	Merck
21.	1,1-diphenyl-2-picrylhydrazyl (DPPH)	Merck
22.	Dragendorff's reagent	Central drug house Pvt Ltd
23.	Ethanol	SD Fine Chem Ltd
24.	Ethyl acetate	Merck
25.	Fehling's solution-A & B	Universal Chemicals and scientific industries South India
26.	Ferric chloride	Merck
27.	Folin-Ciocalteu reagent	Suvidhinath Laboratories, Vadodora
28.	Formic acid	Kanton laboratories
29.	Glycerine	Central drug house Pvt Ltd
30.	Hager's reagent	Kanton Laboratories
31.	Hydrazine hydrate	Central drug house Pvt Ltd
32.	Hydrochloric acid	Universal Chemicals and scientific industries

		South India
33.	Iodine	Universal Chemicals and Scientific industries South India
34.	Isoamyl alcohol	Kanton Laboratories
35.	Iso- propanol	Kanton Laboratories
36.	Lead acetate	Universal Chemicals and Scientific industries South India
37.	Magnesium powder	Central Drug House Pvt Ltd
38.	Mayer's reagent	Kanton Laboratories
39.	Mercuric chloride	Central Drug House Pvt Ltd
40.	Methanol	Central Drug House Pvt Ltd
41.	Millon's reagent	Kanton laboratories
42.	Molisch reagent	Kanton Laboratories
43.	N-hexane	Central Drug House Pvt Ltd
44.	Ninhydrin's reagent	Merck
45.	Nitric acid	Central Drug House Pvt Ltd
46.	Petroleum ether	Loba Chemie Pvt Ltd
47.	Phenol	Universal Chemicals and Scientific industries South India
48.	Phloroglucinol	Universal Chemicals and

		Scientific industries South India
49.	Picric acid	Merck
50.	Potassium dichromate	Central Drug House Pvt Ltd
51.	Potassium permanganate	Central Drug House Pvt Ltd
52.	Potassium ferricyanide	Central Drug House Pvt Ltd
53.	Potassium hydroxide	Medilise Chemicals, Kannur
54.	Pyridine	Central Drug House Pvt Ltd
55.	Safranin	Central Drug House Pvt Ltd
56.	Silica gel for column chromatography	Central Drug House Pvt Ltd
57.	Silver nitrate	Central Drug House Pvt Ltd
58.	Sodium bicarbonate	Universal Chemicals and Scientific industries South India
59.	Sodium carbonate	Central Drug House Pvt Ltd
60.	Sodium hydroxide	Central Drug House Pvt Ltd
61.	Sodium nitrite	Universal Chemicals and Scientific industries South India

62.	Sodium nitroprusside	Merck
63.	Sodium picrate	Central Drug House Pvt Ltd
64.	Sodium dodecyl sulphate (SDS)	Central drug house Pvt Ltd
65.	Starch	Central Drug House Pvt Ltd
66.	Sulphuric acid	Universal Chemicals and Scientific industries South India
67.	Tannic acid	Spectrum Reagents and Chemicals, Kochi
68.	Toluene	Merck
69.	Wagner's reagent	Kanton Laboratories
70.	Zinc powder	Central Drug House Pvt Ltd

8.2 LIST OF BIOLOGICALS

Sl No.	Biologicals	Manufacturer
1.	Bovine albumin serum	ProLiant biologicals
2.	*Candida albicans*	American Type Culture Collection(ATCC),
3.	*Staphylococcus aureus*	

8.3 LIST OF DRUGS

Sl No.	Drugs	Manufacturer
1.	Clotrimazole	Sigma- Aldrich
2.	Diclofenac sodium	Sigma- Aldrich

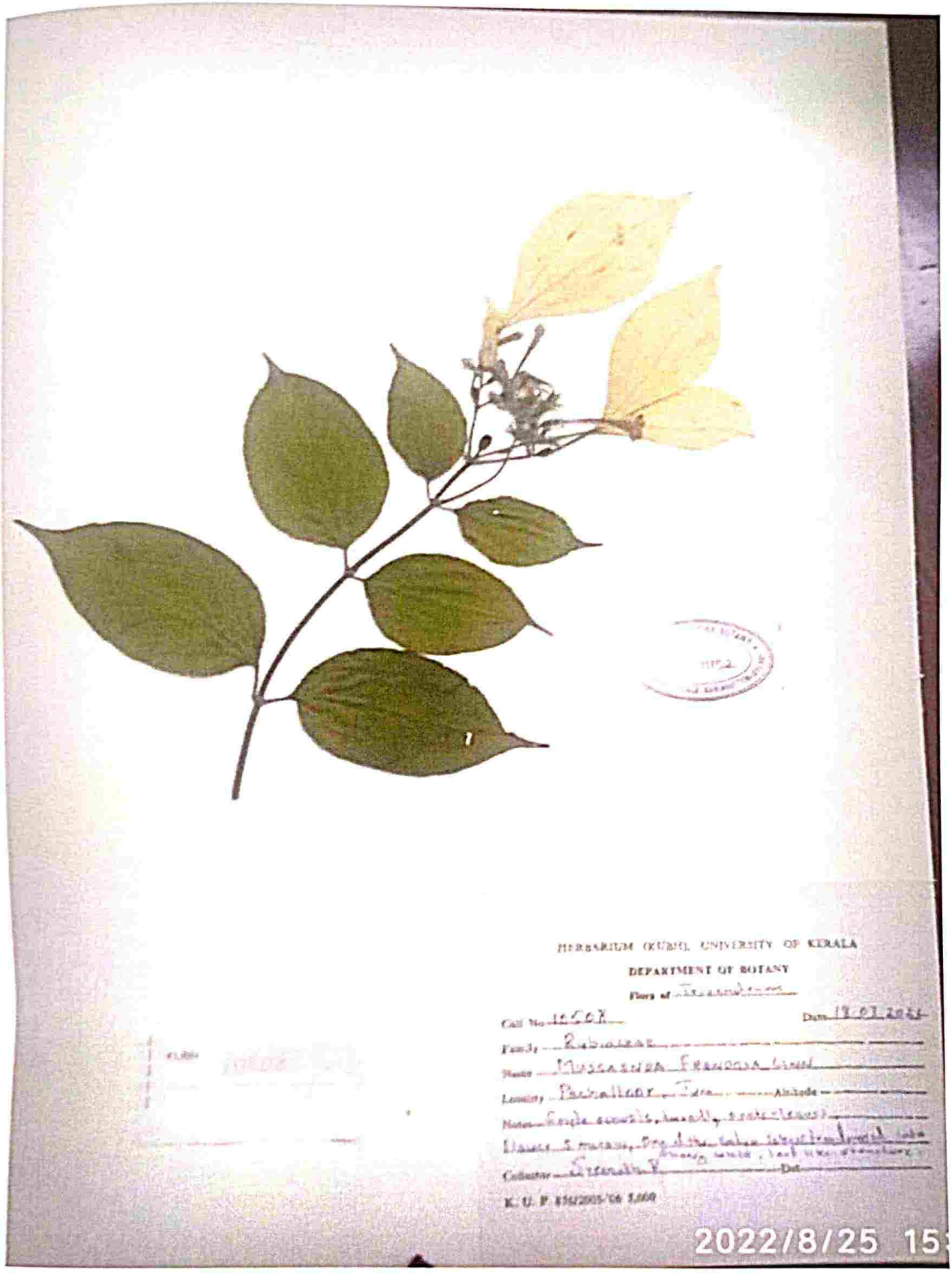
3.	Gallic acid	Loba chemicals
5.	Quercetin	Tokyo chemical industries japan (TCI)
6.	Streptomycin	Sigma- Aldrich
7.	Tannic acid	Sigma- Aldrich

8.4 LIST OF EQUIPMENT'S

Sl No.	Equipment's	Manufacturer
1.	Electronic balance	Shimadzu, Japan
2.	Digital PH meter	Labtronics, India
3.	Hot plate	Lab line instruments, India
4.	Water bath	Medico instruments, India
5.	Hot air oven	Lab line instruments, India
6.	UV -visible spectrophotometer	Hitachi model
7.	FTIR spectrophotometer	Jasco - FT- IR, 4100
9.	Microscope	Labomed
11.	Heating mantle	Lab line instruments, India
12.	Centrifuge	KEMI, India
13.	Micro pipette (100-1000 µl)	Eppendorf, Hamburg, Germany
14.	Soxhlet apparatus	Bionics
15.	Pulverizer	Ken star mixer
16.	U V fluorescence chamber	Ikon instruments
17.	Muffle furnace	Fourtech, Mumbai
18.	5% CO2 incubator	Galaxy® 170 Eppendorf, Germany

19.	Autoclave	Bionics scientific technologies, India

HERBARIUM


HERBARIUM (KUBH), UNIVERSITY OF KERALA
DEPARTMENT OF BOTANY
Flora of
Coll No 1650
Date 19.03.2021
Family Rubiaceae
Name Mussaenda frondosa Linn.
Locality Parathodu, Idukki
Altitude
Notes Fruits ovoids, small, ripe-green
Flowers white, Drupe tube, large, fruit with persistent calyx
Collector
Det.
K. U. P. 856/2005-06 5,000
2022/8/25 15

AUTHENTICATION CERTIFICATE

UNIVERSITY OF KERALA

(Established as University of Travancore by the Travancore University Act in 1937 and reconstituted as University of Kerala by the Kerala University Act of 1957 and presently governed by the Kerala University Act of 1974 passed by the Kerala Legislative Assembly)

DEPARTMENT OF BOTANY

(Re-Accredited by NAAC with 'A++' Grade)

KARIAVATTOM
THIRUVANANTHAPURAM - 695 581
KERALA, INDIA
Phone: (O) 0471-2308301
TELE - FAX: 0471-2308301
Web site : www.keralauniversity.edu
E-mail : botany@keralauniversity.ac.in

No. BOT/KUBH/110/22

Date.....

CERTIFICATE

Certified that the plant material collected and supplied by **Mr. Sreenath R.**, M. Pharm student, College of Pharmaceutical Sciences, Medical College, Thiruvananthapuram, is identified and the voucher specimen is kept in the Herbarium of Department of Botany (KUBH), University of Kerala, Kariavattom as detailed below: -

Samples	Name of the Taxon	Family	English/Local Names	Voucher/Accession No.
1.	*Mussaenda frondosa* L.	Rubiaceae	Eng: Flag bush, White flag Local: Vellila	KUBH 11152

Kariavattom

25.08.2022

Dr. E. A. Siril

Professor & Head

PROFESSOR AND HEAD
Department of Botany
University of Kerala, Kariavattom
Thiruvananthapuram - 695 581